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REVIEW

Initiation and Maintenance of Gastric Cancer: A Focus on CD44 Variant Isoforms and Cancer Stem Cells

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SUMMARY

Cell surface adhesion molecule CD44 has been identified as a gastric cancer stem cell marker. CD44 variant isoforms are expressed abundantly in epithelial-type carcinomas and are associated with initiation and progression of gastric cancer.

Gastric cancer is the third most common cause of cancer-related death. Although the incidence of gastric cancer in the United States is relatively low, it remains significantly higher in some countries, including Japan and Korea. Interactions between cancer stem cells and the tumor microenvironment can have a substantial impact on tumor characteristics and contribute to heterogeneity. The mechanisms responsible for maintaining malignant cancer stem cells within the tumor microenvironment in human gastric cancer are largely unknown. Tumor cell and genetic heterogeneity contribute to either de novo intrinsic or the therapy-induced emergence of drug-resistant clones and eventual tumor recurrence. Although chemotherapy often is capable of inducing cell death in tumors, many cancer patients experience recurrence because of failure to effectively target the cancer stem cells, which are believed to be key tumor-initiating cells. Among the population of stem cells within the stomach that may be targeted during chronic *Helicobacter pylori* infection and altered into tumor-initiating cells are those cells marked by the cluster-of-differentiation (CD)44 cell surface receptor. CD44 variable isoforms (CD44v) have been implicated as key players in malignant transformation whereby their expression is highly restricted and specific, unlike the canonical CD44 standard isoform. Overall, CD44v, in particular CD44v9, are believed to mark the gastric cancer cells that contribute to increased resistance for chemotherapy- or radiation-induced cell death. This review focuses on the following: the alteration of the gastric stem cell during bacterial infection, and the role of CD44v in the initiation, maintenance, and growth of tumors associated with gastric cancer. *(Cell Mol Gastroenterol Hepatol 2017;4:55–63; http://dx.doi.org/10.1016/j.jcmgh.2017.03.003)*

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Gastric cancer is the fifth most common cancer worldwide and the third most common cause of cancer-related death.[1] The incidence of gastric cancer in the United States is relatively low as a result of the diagnosis and treatment of the major risk factor *Helicobacter pylori*.[2] However, the 5-year survival rate for patients diagnosed with this malignancy is only 29%.[3] Importantly, the incidence of gastric cancer varies throughout the world. For example, it is 4 times more common in Japan than in the United Kingdom and occurs at a younger age.[4] A variety of etiologic factors contribute to this higher incidence in countries such as Japan, including *H pylori* prevalence and virulence,[5,6] as well as dietary[7] and genetic variations[8,9] among these populations. Given the poor response of gastric cancer to various existing treatment modalities, there is an unmet need for approaches to predict individual therapy responses. Solid tumors consist of not only malignant cells but also various types of stromal cells, fibroblasts, endothelial cells, and hematopoietic cells such as macrophages and lymphocytes (reviewed by Quante and Wang[10] and Quante et al[11]). Interactions between cancer stem cells and the tumor microenvironment can have a substantial impact on tumor characteristics and contribute to heterogeneity. Heterogeneity contributes to tumor recurrence.[12] Although chemotherapy often is capable of inducing cell death in tumors, many cancer patients experience recurrence because of failure to effectively target the cancer stem cells, which are believed to be key tumor-initiating cells.[13,14] These cancer stem cells are responsible for the formation, maintenance, and continued growth of the tumor,[14,15] and thus highlights the need to target cancer stem cells during treatment. The mechanisms responsible for maintaining malignant cancer stem cells within the tumor microenvironment in human gastric cancer are largely unknown.

The Correa et al[16] model reported that gastric atrophy (parietal cell loss) was one of several significant changes that occurred after chronic inflammation. We now understand that the major cause of chronic inflammation in the normal, acid-secreting stomach is *H pylori* bacterial colonization.[17] It is widely accepted that inflammation that is

Abbreviations used in this paper: Cag, cytotoxin-associated gene; CD, cluster-of-differentiation; CD44v9, CD44 variant isoform containing exon v9; CSC, cancer stem cell; Lgr5, leucine-rich, repeat-containing, G-protein–coupled receptor 5; MDSC, myeloid-derived suppressor cell; PDL1, programmed cell death 1 ligand; PDTX, patient-derived tumor xenograft; ROS, reactive oxygen species; SPEM, spasmolytic polypeptide expressing metaplasia; xCT, SLC7A11.

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caused by *H pylori* infection is a trigger for the development of gastric cancer. An explanation for the causal role of *H pylori* infection in the pathogenesis of gastric cancer has been described by disruption of differentiation of epithelia as a consequence of altered gastric stem cell phenotype.[18,19] The chronic nature of *H pylori* gastritis is critical to the carcinogenic potential of this infection. The long-term interaction of the bacteria and inflammatory mediators with gastric epithelial, progenitor, and stem cells, results in the accumulation of mutations, epigenetic modifications, and deregulation of cell function that ultimately may lead to cancer.[19–21] Therefore, *H pylori* infection plays a critical role during the initiating steps of gastric cancer.

Initiation of Gastric Cancer

Alterations in Epithelial Gastric Stem Cells

Abnormal differentiation (metaplasia) is associated with cancer and may reflect the permanent alteration in the behavior of the stem cells, thus making the gastric stem cell a candidate *H pylori* target. It is hypothesized that tumors develop because of a rare subpopulation of cells (known as cancer stem cells [CSCs]).[10] Although the origin of gastric cancer stem cells remains uncertain, there are a number of key studies that show the expansion of gastric stem cells during bacterial infection that may lead to their alteration and transformation into tumor-initiating cells.[19–21] Among the populations of stem cells within the stomach that may be targeted during bacterial infection, that may lead to metaplasia or aberrant epithelial cell proliferation and differentiation, are cells expressing the leucine-rich, repeat-containing, G-protein–coupled receptor 5 (Lgr5) and the cluster-of-differentiation (CD)44 cell surface receptor.[19–22] Troy marks a specific subset of chief cells that are capable of replenishing entire gastric units in response to injury.[23] In addition, the Sox2+ stem cell compartment has been shown to be critical for normal tissue regeneration,[24] and villin+ is a quiescent stem cell population that becomes apparent upon cytokine stimulation.[25] The expansion of Troy+, Sox2+, and villin+ cell populations in response to *H pylori* infection has not been investigated thoroughly.

Lgr5, located in adult stem cells at the base on the antral glands of the stomach, are capable of long-term renewal of the epithelium.[26] By using lineage tracing to mark cells derived from Lgr5+ stem cells, Sigal et al[19] analyzed the response of these gastric stem cells to *H pylori* infection. The investigators showed that the bacteria formed distinct microcolonies deep in the stomach glands where infection accelerated Lgr5+ stem cell proliferation.[19] The findings show that *H* pylori can colonize and manipulate the gastric stem cell compartment and this has significant implications for *H pylori*–induced gastric disease. These studies also suggest that alterations to stem cells may be responsible for the development of gastric cancer. In support of this idea, human studies have shown that there is enhanced Lgr5 expression in patients with progressive dedifferentiation and metastasis of gastric cancer.[27,28]

In another study, the investigators deleted Smad4 and PTEN in murine gastric Lgr5+ stem cells by the inducible Cre–LoxP system. In mice with altered/mutant Lgr5+ stem cells there was a rapid onset and progression from adenoma to invasive intestinal-type gastric cancer in the antrum.[29] Moreover, it has been shown in an organoid culture system that Lgr5+ gastric stem cell homeostasis is regulated by the Notch signaling pathway.[30] In this study, it also was shown that chronic activation of Notch within gastric Lgr5+ stem cells induced the development of antral polyps in mice, implicating this pathway in gastric tumorigenesis.[30] In addition, GLI2A is an activator form of the Hedgehog transcription factor GLI2, and its expression within Lgr5+ gastric stem cells drives the rapid development of gastric adenocarcinoma.[20] Thus, alterations in Lgr5+ gastric stem cells, which potentially are induced by *H pylori* infection, may be an initiating event for the development of gastric cancer, indicating the potential of Lgr5 as an early diagnostic and prognostic biomarker.

Another host molecule that may influence carcinogenesis in conjunction with *H pylori* is CD44. CD44 is a cell surface adhesion molecule that is expressed on a variety of cells, including gastric epithelial cells, that recently was identified as a gastric cancer stem cell marker, whereby cells expressing CD44 have been shown to possess the properties of cancer stem cells.[31] Defined as a unique subpopulation in tumors that possess the ability to initiate tumor growth and sustain tumor self-renewal, a subpopulation of CD44-positive cells showed spheroid colony formation in serum-free media in vitro as well as tumorigenic ability when injected orthotopically into stomachs of immunodeficient mice in vivo. In addition, the CD44-positive gastric cancer cells showed the stem cell properties of self-renewal, and CD44 knockdown by short hairpin RNA resulted in reduced spheroid colony formation and tumors in immunodeficient mice.[31] In the normal stomach, CD44 labels a population of undifferentiated cells in the isthmus region where stem cells are known to reside.[22] Atrophy of parietal cells that is induced by *Helicobacter* infection or tamoxifen treatment results in the expansion of CD44-positive cells into the base of the gastric glands.[22]

Alternative messenger RNA splicing produces CD44 variant isoforms that are expressed abundantly in epithelial-type carcinomas, although the standard CD44 isoform is expressed predominantly in hematopoietic cells and normal epithelial cell subsets (Figure 1).[32,33] The involvement of CD44 variant isoforms in gastric cancer has not been well studied. What is known, however, is that early studies by Heider et al[34] showed that the normal epithelium expresses 2 of 12 CD44 variant RNAs containing exons V5 and V6. Intestinal-type tumors express a more complex pattern of amplification products that hybridized to exons V5 and V6. In the sample of a diffuse-type tumor, expression of exon V5, but not V6, could be detected.[34] However, CD44 variant isoform containing exox v9 (CD44v9) also has been detected as a potential predictive marker for recurrence in multiple early gastric cancers.[35]

CD44 variant isoforms, in particular the isoform containing exon v6 (CD44v6), was identified as a marker for invasive intramucosal carcinoma and premalignant lesions.[36] Moreover, in cases of sporadic and hereditary

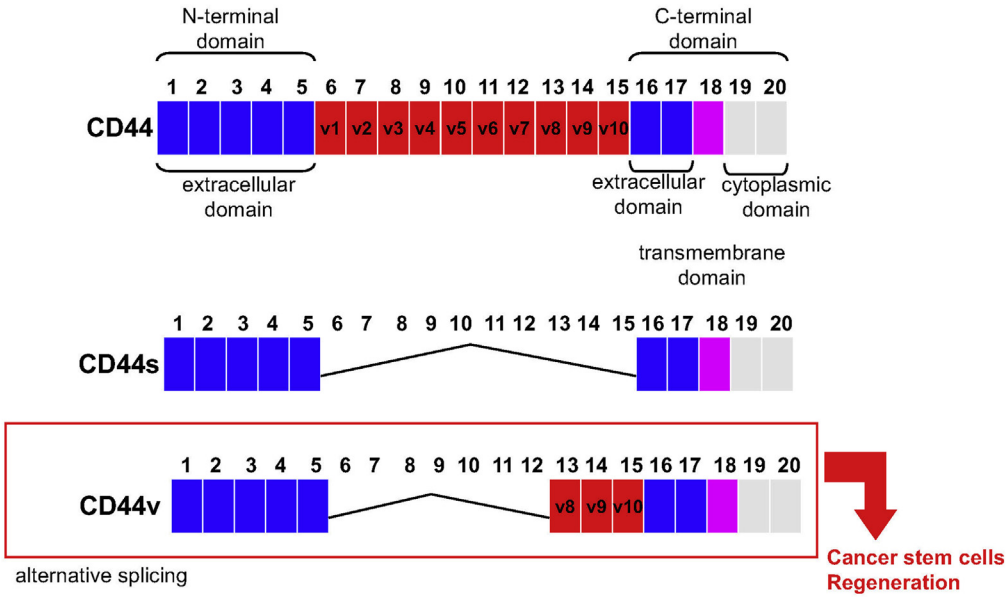

Figure 1. CD44 variant isoforms contain insertions close to the transmembrane region that are generated by messenger RNA splicing. Although the standard CD44 isoform (CD44s) is expressed predominantly in hematopoietic cells and normal epithelial cell subsets, CD44v8–10 is identified as a cancer stem cell marker. Recently, CD44v9 was shown to emerge during gastric regeneration.

diffuse gastric cancer, CD44v6 expression correlated inversely with the expression of E-cadherin.[36] Another variant isoform, CD44v8–10, has been shown to be a cancer stem cell marker in primary gastric cancer.[37] We have observed an increase in CD44v9 expression in the stomachs of patients infected with *H pylori* (unpublished data). In addition, the higher expression for CD44v9 was observed in gastric cancer tissues, with greater expression rates for CD44v9 in the intestinal type or well-differentiated gastric cancer than in the diffuse type or poorly differentiated gastric cancer.[38,39] Within cancer cells, CD44v9 interacts with the glutamate-cysteine transporter SLC7A11 (xCT), stabilizes the protein, and thereby potentiates defense against reactive oxygen species that subsequently promotes

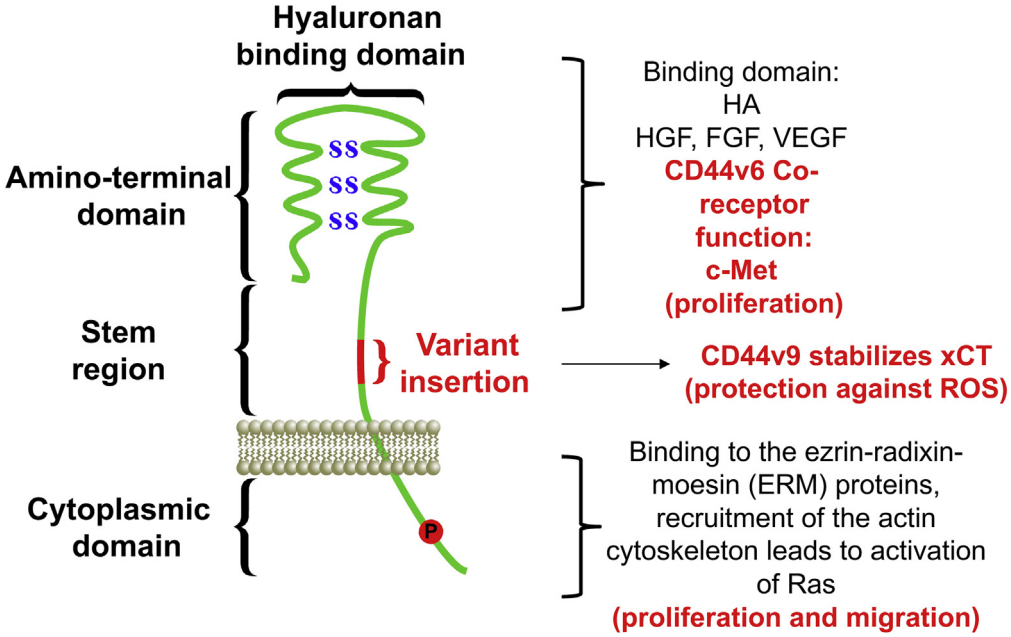

Figure 2. Structure, binding sites, and interactions of CD44. The CD44 protein is composed of an extracellular N-terminal domain, a stem region, and the carboxyl terminal cytoplasmic domain/tail. The stem region, close to the transmembrane region, is the site of variant exon product insertion. The N-terminal domain contains highly conserved disulfide bonds (SS) that are essential for hyaluronan (HA) binding. The C-terminal cytoplasmic tail contains several phosphorylation (P) sites that regulate the interaction of CD44 with the cytoskeletal linker proteins for the regulation of cell proliferation and importantly migration. Note that CD44v6 acts as a co-receptor for c-Met signaling and c-Met ligands including hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) to regulate proliferation. CD44v9 stabilizes the cysteine–glutamate transporter xCT for the protection against ROS and subsequent cell survival and proliferation.

tumor growth[40] (Figure 2). Thus, CD44 and its splice variants are associated positively with the initiation and progression of gastric cancer and may play important roles in the diagnosis, therapy, and prognosis of this disease.

Notably, CD44v6 acts as the co-receptor for c-Met.[41,42] The extracellular domain of CD44v6 is necessary for c-Met activation, which is dependent on hepatocyte growth factor binding[41] (Figure 2). The co-receptor function of CD44v6 for c-Met is of particular interest given that studies pinpoint CD44v6 as a marker of early invasive intramucosal gastric carcinoma.[36] The cytotoxin-associated gene (Cag) pathogenicity island is a strain-specific constituent of *H pylori* that augments cancer risk.[43] The Cag pathogenicity island encodes a type IV secretion system that is a multimolecular complex that mediates the translocation of bacterial factors into the host cell.[43,44] Evidence in the literature shows that *H pylori*–expressed CagA accumulates in gastric cancer cells specifically expressing CD44 and showing suppression of autophagy by their resistance to reactive oxygen species (ROS), and thus suggesting that CD44+ cells are resistant to oxidative stress.[45] In cells lacking CD44 expression, CagA is degraded by autophagy induced by the accumulation of ROS.[45] Upon delivery into the host cells by the type IV Cag secretion system, CagA translocates into the host cell cytoplasm where it can stimulate cell signaling through interaction with several host proteins,[43,46,47] including the tyrosine kinase c-Met receptor.[48–50] CagA exerts effects within host cells by inducing hyperproliferation and disrupting apical-junctional complexes and cellular polarity.[51–53] Suzuki et al[54] showed that CagA CM motifs interact with Met, leading to sustained PI3K-AKT signaling in response to *H pylori*, leading to β-catenin activation and cellular proliferation. We have published that CD44v6 acts as a co-receptor for the function of c-Met in response to *H pylori* infection and bacterial-induced epithelial proliferation.[21] Collectively, these studies suggest that CD44 and its variant isoforms may not simply be markers of the gastric cancer stem cell, but also actively involved in the initiation and progression of disease.

Unregulated Spasmolytic Polypeptide/Trefoil Factor 2–Expressing Metaplasia in the Initiation of Gastric Cancer

It is accepted that loss of acid-secreting parietal cells is a prerequisite for the development of metaplasia and a mucosal lineage change associated with increased risk for gastric cancer.[55–57] Loss of parietal cells results in the transdifferentiation of the chief cell lineage into a mucous cell metaplasia identified as spasmolytic polypeptide expressing metaplasia (SPEM).[58] Although *Helicobacter* infection, tamoxifen treatment, and parietal cell–specific protonophores (DMP-777 and L635) are known inducers of SPEM,[58,59] parietal cell loss alone is not sufficient to induce metaplasia.[60] In a study using a mouse model expressing the diphtheria toxin receptor specifically in parietal cells to induce their death, metaplastic reprogramming of chief cells was not observed, suggesting mechanisms beyond parietal cell injury and apoptosis.[60] Metaplastic mucous cells arising for the loss of parietal cells express trefoil factor 2, also known as spasmolytic polypeptide, thus leading to the designation of this lineage as SPEM.[56,61,62] SPEM also is associated with increased expression of cell surface protein CD44, in particular CD44v9.[63] SPEM also has been identified in the mucosa surrounding intestinal-type gastric cancers in human beings.[62] Importantly, mice infected with *Helicobacter felis* for more than 12 months showed progression of the metaplasia to dysplasia.[56,57,64] These reports indicated that metaplastic glands induced by parietal cell loss and chronic inflammation progress toward dysplasia. Interestingly, we reported the induction of SPEM after gastric injury.[65] SPEM was identified in the ulcer margin in the regenerating gastric glands and disappeared when the mucosa returned to its normal compendium of cell lineages, suggesting a possible role for SPEM in ulcer repair.[65] Collectively, these studies suggest that in response to gastric ulceration, SPEM is a regulated mechanism that may contribute to repair. SPEM in the setting of parietal cell atrophy and chronic inflammation is an unregulated precursor lineage for the development of dysplasia associated with gastric cancer development. The stem cell marker CD44v9 also marks SPEM and may contribute to the production of metaplasia.

Maintenance of Gastric Cancer: The Role of the Cancer Stem Cells

The gastrointestinal tumor microenvironment is required for tumor initiation, progression, and metastasis. Solid tumors are heterogeneous and consist of cancer cells, cancer stem cells, and various types of stromal cells, fibroblasts, endothelial cells, and hematopoietic cells, mainly macrophages and lymphocytes.[10,11,40,66] Poor response of gastric cancer to various existing treatment modalities may be accounted for by the cellular heterogeneity of the tumor microenvironment. In particular, chemotherapy is one of the standard methods of treatment in many cancers including gastric cancer.[67] Although chemotherapy often is capable of inducing cell death in tumors, many cancer patients experience recurrence because of failure to effectively target the cancer stem cells, which are believed to be key tumor-initiating cells. One CSC model proposes that the growth of the tumor is driven by a population of self-sustaining cells with stem cell properties of proliferation and an ability to differentiate into the entire heterogeneous population of the tumor.[13,14] These CSC are responsible for the formation, maintenance, and continued growth of the tumor.[14,15] This model highlights the need to target CSCs with chemotherapy. The targeted agent eliminates the chemoresistant CSC population, preventing recurrence of the tumor, while the chemotherapy targets the differentiated cells. As discussed earlier, variant isoforms of CD44 including CD44v6 and CD44v9 have been reported to have prognostic value in gastric cancer. For example, CD44v9 expression in primary early gastric cancer is a predictive marker for recurrence,[35] and the presence of CD44v9-positive circulating cancer cells is associated strongly with recurrence and poor survival rates in colorectal cancer.[68] In our laboratory, gastric

organoids derived from the tumor tissue of a patient with diffuse-type gastric cancer expressing CD44v9 were resistant to cisplatin. Interestingly, inhibition of xCT by sulfasalazine sensitized the organoids to cisplatin-induced atrophy (unpublished data). Thus, this is an example by which therapy targeted to the CD44v9–xCT system may impair the ROS defense by cancer stem cells and thereby sensitize them to currently available treatments.

Immune suppression and adaptive immune resistance are other mechanisms that contribute to the maintenance and growth of tumors. The immune system typically detects and eliminates cancer development via a process termed *immune surveillance*.[69–71] However, during immune evasion, the tumor cells escape the immune system. For example, tumor-derived cytokines can induce the differentiation of immune effectors to a suppressive phenotype such as tumor-associated macrophages, myeloid-derived suppressor cells (MDSCs), and regulatory CD4+ T cells.[72] Tumor-associated macrophages within the tumor microenvironment have been described as protumorigenic, supporting cancer initiation and progression.[71,73–75] Another example is the suppressive function of MDSCs. MDSCs are a heterogeneous myeloid cell population that infiltrates tissue in response to infection, injury, autoimmune disease, and cancer.[76] A study by the Merchant laboratory identified Schlafen 4 (*Slfn4*) as a GLI1 target gene and myeloid differentiation factor that correlated with the development of SPEM in mice.[77] A recent study by the same group then showed that migration of Slfn4-expressing cells from the bone marrow to the stomach in response to *Helicobacter* infection showed MDSC markers, and acquired the ability to inhibit T-cell proliferation.[78] This supports MDSC suppression of T-cell function and subsequent dampening of the immune response to create a microenvironment favoring tumor growth.[79]

Tumor cells may evade the immune response by inducing T-cell inactivation. Dendritic cells within the tumor microenvironment present tumor-specific antigens on major histocompatibility complex class I to CD8+ T cells that then can prime an antigen-specific T-cell response.[80] In colorectal tumors, high densities of cytotoxic and memory T cells in the tumor microenvironment are associated with reduced recurrence of disease.[81] Tumors can evade immune surveillance by expressing molecules such as programmed cell death 1 ligand (PDL1), which interacts with PD1 and subsequently inhibits CD8+ cytotoxic T-lymphocyte proliferation, survival, and effector function[82–84] (Figure 3). In addition, CTLA-4, which is expressed minimally on the surface of resting T lymphocytes, is highly expressed on activated T lymphocytes.[85,86] Importantly, ligands B7-1/B7-2, expressed on tumor cells, bind to CTLA-4 and inhibit effector T-cell function[85,86] (Figure 3). Although anti-PD1 antibodies are already in clinical trials for gastric cancer treatment,[87] whether PD1-PDL1 or CTLA-4/B7 interactions inhibit CD8+ cytotoxic T-cell effector function within the gastric tumor microenvironment has not been well studied. Importantly, a preclinical model that predicts the efficacy of such targeted therapies in individual patients with gastric cancer does not exist. In support of this notion, however, PDL1+ (B7-H1+) gastric cancer stem cells show an increased proliferative capacity.[88] PDL1 also has been identified as an independent prognostic factor for patients

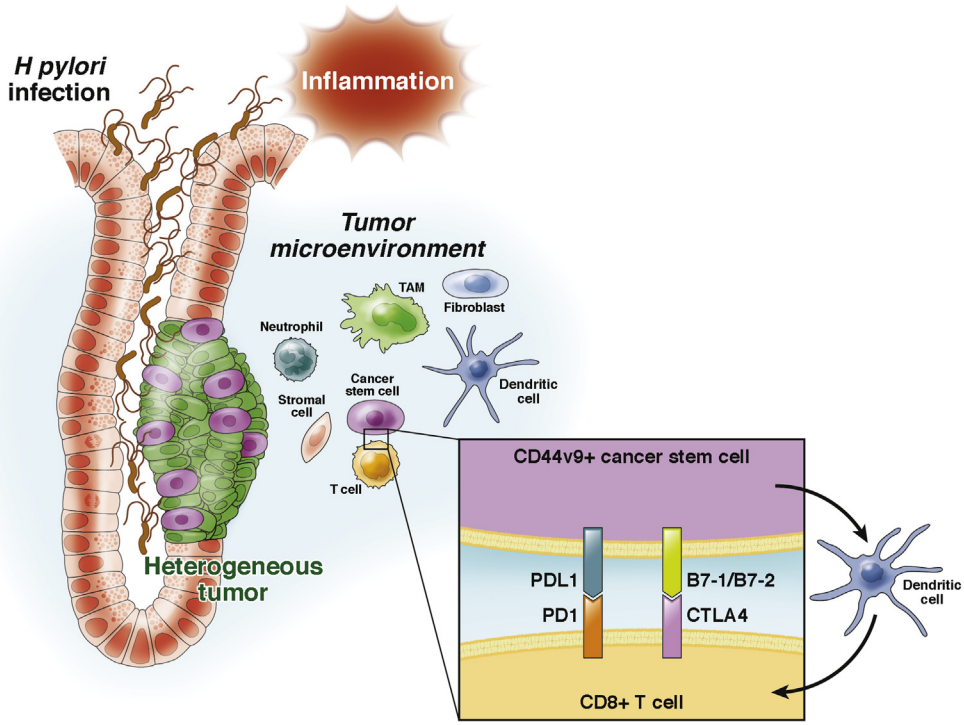

Figure 3. Illustration for the proposed development of gastric cancer initiated by chronic inflammation. During *H pylori* infection, CD44v9 expression emerges, a marker of the gastric cancer stem cell. Despite loss of *H pylori* infection over time, CD44v9 expression is maintained in cancer stem cells within the tumor. We may predict that tumor antigen secreted by the CD44v9+ cells activate dendritic cells that subsequently activate CD8+ cytotoxic T cells to express PD1 and CTLA-4, a mechanism by which cancer stem cells can evade the immune response via inactivation of T-cell effector function. TAM, tumor-associated macrophages.

with stage II/III gastric cancer, suggesting that patients with stage II/III gastric cancer might be appropriate for PD1/PDL1-targeted therapy.[89] Furthermore, selective expression of the PDL1 was observed on CD44(+) cells in squamous cell carcinoma of the head and neck[90] and breast cancer cells.[91] In addition, PDL1 expression was associated with worse overall survival in patients with stage II/III gastric cancer, suggesting that these patients might be appropriate for PD1/PDL1-targeted therapy.[89]

Future Directions

Given the poor response of gastric cancer to various existing treatment modalities, there is an unmet need for approaches to predict individual therapy responses. Although cancer cell lines and patient-derived tumor xenografts (PDTXs) have proven very valuable in fundamental cancer research, both models have disadvantages. Cell lines lack the tumor heterogeneity found in tumor tissues, and although PDTXs bear promise as preclinical models for human cancer, there are several limitations. For example, similarities between PDTX and parental tumors cannot be assumed until rigorously tested, tumor–host interactions are not always conserved across species and tumor immunity is entirely absent. As mentioned earlier, a preclinical model that predicts the efficacy of such targeted therapies in individual patients with gastric cancer does not exist. We and others have shown that gastric organoids not only have the tissue architecture and physiological function of the human stomach, but are also an experimentally tractable system allowing for cell and genetic manipulation.[21,30,65,92,93] Moreover, patient-derived gastric tumor organoids can be transplanted orthotopically into NOD.Cg-Rag1[tmMom]/L2rg[tmWjl]Tg(CMV-IL3, CSF2, KITLG)/Eav/J (NRGS) (NRG-SGM3) mice expressing human-derived immune cells so that the role of the immune response within the tumor microenvironment may be identified in vivo (unpublished data). Understanding the immune response within the tumor microenvironment is crucial not only to addressing important biological questions, but also for the success of current and future immunotherapy. Potential studies that use organoids include the following: (1) in vitro and in vivo organoid-based approaches for the study of the interaction between cancer stem cells and the immune microenvironment using organoid/immune cell co-cultures, (2) a preclinical organoid-based platform for anticancer drug evaluation, and (3) in vitro and in vivo preclinical models that will allow us to effectively evaluate novel cancer therapeutics as well as to identify predictive biomarkers for gastric cancer. Although organoids have been widely used for biological and clinical studies, this system does show key challenges, as follows: (1) the lack of the native microenvironment, (2) organoids within the same culture may be phenotypically heterogeneous, and (3) maintenance of epithelial heterogeneity within the organoid culture over time is unclear. Despite these challenges, the development of cutting edge in vitro and in vivo organoid-based approaches from a common patient sample is a critical first step for personalized medicine providing a preclinical approach to prevent, diagnose, or treat gastric cancer.

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