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TOPIC HIGHLIGHT

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Cancer stem cell hypothesis and gastric carcinogenesis: Experimental evidence and unsolved questions

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Abstract

Traditionally, the clonal evolution model has been used to explain gastric cancer (GC) growth dynamics. According to this model, GC cells result from multiple mutations over time resulting in a population of continually diversifying cells. This heterogeneity enables the survival of different clones under particular conditions allowing growth at metastatic locations or resistance to chemotherapeutics. Cancer stem cell (CSC) theory completely overturns this traditional understanding of cancer suggesting that only CSCs can self-renew and promote tumor growth. CSCs are relatively refractory to conventional therapies, thus explaining why anti-cancer therapies are far from curative and why relapses of cancer are frequent. The identification of the CSC component of a tumor might, thus, open new therapeutic perspective based on the selective targeting of this small population of cells. In this review we examine the current scientific evidence supporting the existence of CSC in gastric tumors and analyze the main unsolved questions of this difficult field of cancer research.

Key words: Cancer stem cells; Gastric cancer; CD133; CD44

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INTRODUCTION

Despite the overall decreasing incidence and mortality rates observed over the last 50 years, gastric cancer (GC) still ranks as the fourth most common malignancy and the second leading cause of cancer-related death, in both sexes, worldwide[1]. About one million new cases and more than 700 000 deaths were reported in 2008, with an overall 5-year survival rate < 20%[2]. The late diagnosis of the disease, generally recognized only at the advanced stage, and the intrinsic resistance to radio- and chemotherapy may account for the poor prognosis of GC. At present, prevention is likely to be the most effective means of reducing the incidence of and mortality from this disease. However, to be successful, this strategy requires knowledge of the etiological factors and pathogenetic mechanisms involved in gastric carcinogenesis.

GC: ONE DEFINITION, DIFFERENT DISEASES

In the recent years, it has become increasingly apparent that the GC is a heterogeneous disease. The anatomical location, according to which GC can be subdivided in proximal or cardia GC and distal or non-cardia GC, identifies diseases that drastically differ in epidemiological distribution, pathologic profile, clinical presentation and prognosis[3,4].

While the incidence of distal GC shows a remarkable downward trend in incidence and mortality rate, proximal tumors have been increasing in incidence with annual rates of up to 4%-5% per year since the 1970s, mainly among males and notably in the UK, Ireland, Northern Europe, Australia and New Zealand, China, and North America[5]. The rate at which the incidence of proximal stomach cancer has risen exceeds that of any other cancer and is taking place principally in countries with relatively low overall rates of GC. In contrast to cancer of the distal part of the stomach, proximal tumors affect the higher social classes and are associated with high BMI and obesity rather than the gastric-specific pathogen *Helicobacter pylori* (*H. pylori*) which represents the primary risk factor for distal GCs[6,7].

According to the historical Laurén's classification, non-cardia GC can further be subdivided into two main histological subtypes, diffuse and intestinal[8]. Intestinal-type GC, the most common variant in populations at high risk, consists of gland-like structures that mimic the glandular architecture of the intestinal tract and is recognized by a series of precancerous lesions, i.e., atrophy, intestinal metaplasia and dysplasia. The diffuse type, relatively more frequent in populations at low risk, lacks any glandular structure and usually arises in the context of a chronic inflammation but without any identifiable histological precursor lesions[9]. While environmental factors such as diet and *H. pylori* infection strongly influence the natural history of intestinal-type GC, the role of environmental factors appears less important than the genetic influences in diffuse-type disease.

"MULTISTEP" PROCESS OF GASTRIC CARCINOGENESIS

The origins of intestinal-type GC and the mechanisms of the development of this type of tumor are quite well characterized. Intestinal-type GC typically arises in the setting of chronic gastritis and develops through the intermediate stages of atrophic gastritis, intestinal metaplasia, dysplasia, and finally cancer[10]. This lengthy process, known commonly as the "Correa pathway", is triggered by *H. pylori* infection and depends on the sustained chronic inflammation of gastric mucosa that in turn fosters a cascade of genotypic events responsible for cancer development[9,11]. This traditional model of gastric carcinogenesis fits the more general theory of carcinogenesis postulated by Nowell and Vogelstein according to which the "morphological" evolution of cancer can been considered the end result of sequential accumulation of mutations in oncogenes and tumor suppressor genes in single cells[12,13]. During tumor progression, transformed cells continue to acquire new mutations with the emergence of clones that out-compete others due to increased proliferative or survival capacity and the emergence of genetically variant sub-lines with more aggressive phenotype[13].

DEFINITION OF CANCER STEM CELLS

Interest in gastric cancer stem cells (CSCs) has recently arisen in the broader context of the CSC hypothesis which represents a modern interpretation of the "embryonal rest theory" developed by Julius Cohnheim in 1867. Based on the observation that cancers are composed of a heterogeneous population of cancer cells very similar to the structure of a normal organ, the CSC theory hypothesizes that cancer derives from a stem cell compartment that undergoes an abnormal and poorly regulated process of organogenesis analogous to many aspects of normal stem cells[14,15]. CSCs share important properties with normal tissue stem cells including self-renewal (by symmetric and asymmetric division) and differentiation into the heterogeneous non-tumorigenic cancer cell types that constitute the bulk of the tumor[16]. CSCs are relatively refractory to therapies that have been developed to eradicate the rapidly dividing cells that constitute the majority of the non-stem cell component of tumors, thus explaining why anti-cancer therapies are far from curative and why relapses of cancer are common[16,17].

The existence of CSCs had been hypothesized for many decades. However. it was not until 1994 that malignant stem cells were isolated from patients with acute myeloid leukemia, in which a rare subset comprising 0.01%-1% of the total population induced leukemia when transplanted into immunodeficient mice[18,19]. Since this first experimental evidence, growing attention has been paid to the identification of a possible CSC in solid tumors.

Do date, two methods are universally recognized to identify CSCs[20,21]. One is an *in vitro* method termed "spheroid colony formation," and the other is an *in vivo* method that involves the implantation of candidate CSCs under the skin or within specific organ sites (e.g., orthotopic) of immunodeficient mice (e.g., NOD/SCID mice, nude mice, Rag2/-C double-mutant mice).

The growth of spherical colonies after a few weeks is considered indicative of self-renewal ability and would be consistent with a CSC phenotype although the growth of cells in immunodeficient mice is needed to demonstrate true tumourigenicity and is generally considered the gold standard for proving the existence of CSCs.

Putative CSCs are usually identified based on the expression of specific surface markers[22]. Among the numerous surface markers currently under investiga-

tion, CD133 and CD44, first identified as specific CSC markers for glioblastoma and breast cancer respectively, have proven to be the most useful for the identification of CSCs in solid tumors[23]. By using this experimental approach the existence of CSCs has been demonstrated in breast cancer[21,24], brain cancer[20,25], prostate cancer[26], melanoma[27-29], colon cancer[30-32], liver cancer[33], and pancreatic cancer[34].

CD133, an apical plasma membrane protein found predominantly on embryonic epithelial structures, was first used to isolate neural CSCs from a range of paediatric brain tumors[25]. Injection of as few as 100 $CD133^+$ cells in NOD-SCID mice produced in the animal model a tumor that was a phenocopy of the patient's original tumor. Later, it was demonstrated that a $CD133^+$ cell population, although accounting for only 2.5% of the tumor cells, included tumor-initiating cells in human colon cancer. Indeed, the subcutaneous injection of as few as 3000 $CD133^+$ cells readily reproduced the original tumor in NOD/SCID mice[31].

CD44 is a class I transmembrane glycoprotein that can act as a receptor for extracellular matrices such as hyaluronic acid and it is a known downstream target of the Wnt/β-catenin pathway[35]. It was the first marker identified for a solid tumor stem cell in a study of tumorigenic breast cancer[24]. In pancreatic cancer, cells expressing CD44, CD24, and ESA had a 100-fold enhanced tumorigenic potential, with injection of as few as 100 triple positive $CD44^+CD24^+ESA^+$ cells resulting in tumor formation in 50% of animals[34].

CSC IN GC: THE EXPERIMENTAL EVIDENCE

First evidence of the existence of a CSC component in GC came from a study by Takaishi and colleagues who analyzed a panel of human GC cell lines (i.e., AGS, NCI-N87, MKN-28, MKN-45 and MKN-74) and identified putative cancer initiating cells within a $CD44^+$ cellular fraction[35]. Consistent with the standard definition of CSCs, $CD44^+$ cells isolated from MKN-45, MKN-74 and N-87 GC cell lines formed spheroid colonies under non adherent conditions in serum-free media and xenograft tumors in the stomach and skin of SCID mice. $CD44^+$ cells exhibited the stem cell properties of self-renewal and the ability to form differentiated progeny of $CD44^-$ cells. In addition, CD44 knockdown reduced the efficiency of spheroid colony formation as well as the size of xenograft tumors. Finally, in a mouse model of *Helicobacter*-dependent gastric carcinogenesis, INS-GAS mice infected with *Helicobacter felis* developed invasive gastric lesions strongly positive for CD44 immunostaining, especially at the invading edge of the tumors.

At the same time, Fukuda *et al*[36] described putative gastric tumor-initiating cells by isolating and characterizing the so-called side population (SP) in five human GC cell lines (MKN45, KATOIII, MKN74, MKN28 and MKN1) and three cases of primary human GCs. SP cells, firstly described by Goodell[37], are a small subpopulation of cells with enriched stem cell activity and a distinctive expression profile of the ATP-binding cassette (ABC) transporter. Due to the presence of ABC transporters, SP cells are characteristically refractory to Hoechst 33342 dye-staining and resistant to certain drugs. They have been isolated from numerous human solid cancers such as lung cancer[38], mesenchimal neoplasms[39], acute myelogenous leukemia[40], neuroblastoma and glioma[41,42].

Using flow cytometry for SP cell isolation, Fukuda *et al*[36] demonstrated a fraction of SP cells, ranging from 0.02% to 1.93%, in all GC cell lines analyzed. Using MKN45 cells, the cell line with the highest percentage of SP cells, they demonstrated stem cell-like characteristics of SP cells based on the ABC transporter gene expression (MDR1 and BCPR1), chemo-resistance and tumorigenicity *in vivo*.

Although the experiments conducted by the Takaishi and Fukuda groups provided evidence for the existence of a CSC component in GC, both groups underlined the need for validation studies based on the analysis of GC specimens. Studies carried out on cancer cell lines cannot be easily generalized to the primary tumors. Indeed, although cell lines share many of the molecular and genetic features of the primary tumors from which they derive, primary tumors have heterogeneous cellular, genetic, and epigenetic characteristics.

By using CD133 and CD44 cell surface markers to enrich the putative CSC fraction, we analyzed tissues from 44 patients who underwent gastrectomy for primary GC. We set out to investigate whether tumors contained a cell subset expressing stem-like properties and whether this subpopulation had tumor-initiating properties by subcutaneous or intraperitoneum injection in two different model of immunodeficient animals (i.e., NOD/SCID and nude mice)[43]. Although all samples analyzed contained a significant percentage of both $CD133^+$ and $CD133^+/CD44^+$ cells, they failed to reproduce cancer in both mice models while the unseparated cells produced glandular-like structures in 70% of the experiments. These results prompted us to conclude that neither $CD133^+$ nor $CD133^+/CD44^+$ isolated from primary human GCs express stem-like properties or exhibit tumor-initiating properties.

UNSOLVED QUESTIONS

CSC markers

The identification of a putative CSC subpopulation with validated methods and markers for each tumor entity remains controversial. The selection of the marker for CSC recognition in solid tumors is a notoriously thorny issue in the field of CSCs[22]. Most markers for sorting are empirical and derive from normal stem cells. In this context, only recently has colon cancer research led to the identification of two related receptors, leucine-rich

repeat-containing G-protein coupled receptors (Lgr) 5 and Lgr6, that are expressed by small populations in a variety of adult organs including the stomach[44]. Lineage tracing studies showed that $Lgr5^+$ cells are actively dividing, multipotent stem cells responsible for the long-term renewal of the entire gastric epithelium[45]. More interestingly, the inactivation of APC in $Lgr5^+$ cells, rapidly initiated tumor formation in the pyloric epithelium in an animal model. Thus, the analysis of $Lgr5^+$cells appears to be promising for the identification of a putative CSC population in primary human GC even though Lgr5 has been described as being expressed in both tumorigenic and non-tumorigenic gastric cell lines[35].

Animal models

CSCs have been defined on the basis of their ability to seed tumors in animal hosts, to self-renew and to spawn differentiated progeny (non-CSCs)[17]. The experiments performed to demonstrate the existence of CSCs are usually carried out in immunodeficient mice. However, it is noteworthy that the type of immunodeficient animal model can affect the result of xenograft transplantation and differences in tumor initiation may be less striking if more highly immunodeficient mice are used. Additional aspects of host biology that can influence cancer cell engraftment rate include vascularization at the site of implantation, extracellular matrix constitution and growth factor availability. These considerations give rise to the thorny issue of choosing an appropriate animal model to measure CSC representation. The ideal animal model would, in fact, accurately represent tumor CSC biology as it occurs in humans. In this context, several animal host models would probably be required, each of which able to recapitulate the specific tissue microenvironment of the human tumor analyzed[46].

Tumor microenvironment

The natural history of GC strongly differs according to anatomic site of origin and histological type. For example, intestinal type GC represents the end result of a multistep process triggered by *H. pylori* infection and evolving in the context of a chronic inflammatory state of the gastric mucosa[10]. Therefore, the absence of an appropriate microenvironment could explain the inability of the putative CSCs to reproduce the structural complexity of the primary gastric tumors when injected under the skin or orthotopically in immunocompromised animal models. It is noteworthy that a sustained chronic inflammation plays an active and primary role in transforming tissue stem cells into tumor cells[47] and GC represents the typical tumor system where synergy between *H. pylori* infection, inflammation and host factors is required for effective carcinogenesis. In addition, murine studies have demonstrated that the induction of preneoplasia correlates better with the type of inflammatory response than with the strain of *H. pylori*, again emphasizing the functional importance of the inflammation compared to bacterial factors[48].

Cellular origin of GC

GC is of epithelial origin and, as reported for epithelial cancers in general, is believed to originate from transformation of tissue stem cells[49]. However, bone marrow-derived cells (BMDCs), which are frequently recruited to sites of tissue injury and inflammation, might also represent a potential source of malignancy in the stomach. Chronic infection of C57BL/6 mice with *Helicobacter* induces repopulation of the gastric mucosa with BMDCs. These cells can progress through metaplasia and dysplasia to intraepithelial cancer[50]. These observations seem to suggest that epithelial cancers can originate from marrow-derived sources. However, a recent report based on the analysis of solid organ neoplasia developed in female recipients of male allogenic stem cell transplants demonstrated that only donor-derived neoplasia-associated myofibroblasts were detected in GCs, thus suggesting that donor BMDCs did not contribute to the neoplastic epithelium in humans[51].

CONCLUSION

The question of gastric tumor origin remains biologically relevant. If correct, the CSC hypothesis would require that we rethink the way we diagnose and treat these tumors, as our objective would have to shift from eliminating the bulk of rapidly dividing, but terminally differentiated components of the tumor, and be refocused on the minority stem cell population that fuels tumor growth.

Over the last 5 years, the rapidly evolving field of CSC research has generated great interest in the potential therapeutic applications and might play a pivotal role in changing how basic cancer researchers, clinical investigators, physicians, and cancer patients view cancer. Therefore, additional investigations intended to evaluate CSC phenotypes by using different markers, different experimental approaches and/or gene expression signatures are advisable to address the difficult subject of gastric CSC.

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