

REVIEW

Tumor angiogenesis in gastric cancer

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Abstract

Growth of solid tumors, including gastric cancer, and the formation of metastasis depend on the induction of new blood vessels; in tumors, angiogenesis is uncontrolled and immature. This is a complex process, depending on a great variety of angiogenic factors, one of the most important being the vascular endothelial growth factor. In order to suppress the tumor development and the occurrence of metastasis, clinical trials have been developed with angiogenesis inhibitors, many of them with encouraging results. Further research is needed in regard to the idea of combined antiangiogenic therapy with conventional chemotherapy, or even immune or genetic therapies, in order to increase treatment efficiency and to suppress side effects.

Keywords: tumor angiogenesis, gastric cancer.

☞ Introduction

In 1971, Folkman advanced the hypothesis according to which tumor growth is angiogenesis-dependent [1], based on initial experiments at the beginning of the 1960s, which revealed that tumor development is severely restricted in perfused organs in the absence of tumor vascularisation [2–4].

It has been suggested that tumor cells and endothelial cells in the neoplasm achieve an ecosystem, and endothelial cells can be activated from a latent status in a phase of rapid growth by a chemical signal released by tumor cells. The possibility of antiangiogenic therapy has also been speculated for the first time [1, 5]. Later on, the area of research in the field of angiogenesis has been expanded, involving nowadays the field of fundamental research, as well as numerous clinical specialties.

Tumor angiogenesis, represented by the formation of new vessels through budding of the pre-existent ones, is essential for tumor growth over the microscopic limit (2 mm) [6]. This phenomenon depends on angiogenic factors with direct action [7], such as the endothelial growth factor, placental growth factor, angiopoietins and angiogenic factors with indirect action (fibroblastic growth factor, endothelial growth factor derived from platelets, interleukins 1, 2, and 8). The intensity of this process can be expressed as intratumoral microvascular density.

Recent research has demonstrated that tumor development and metastasis requires constant neoangiogenesis, which can ensure a path of hematogenous metastasizing, influencing patient prognosis [8]. The angiogenesis process is the result of the balance between a series of angiogenic factors and angiogenesis inhibitors. In normal tissues, angiogenesis

is strictly controlled [9, 10], while in tumoral tissue it is an uncontrolled and immature process [11].

Numerous studies have been performed regarding the important role of angiogenesis in the growth, invasion, metastasizing, and recurrence of gastric cancer (GC).

☞ The expression of the endothelial growth factor (VEGF) and intratumoral vascular microdensity (IVM) in GC

Dvorak has shown for the first time an association between tumoral angiogenesis and the increase in microvascular permeability, which has led to identifying the vascular permeability factor (VPF) [12], proved subsequently by Ferrara to be a specific inducer of angiogenesis named the endothelial growth factor (VEGF) [13].

VEGF is a mitogenous and mobilizer of endothelial cells, with an angiogenic effect *in vivo* [14–16]. Its expression is correlated with blood vessel growth during embryogenesis [17–19], with embryogenesis at the female genital tract and with tumor development [20–23].

VEGF is a homodimeric protein of 40–45 kDa, secreted by a wide variety of cells and by most tumoral cells, existing in five different isoforms (VEGF – A, B, C, D, and E) and presenting three receptors (Flt-1 and KDR) specific for endothelial cells [24, 25]. VEGF-C binds to Flt-4, expressed preferentially by the lymphatic endothelium [26, 27]. The expression of VEGF is stimulated by hypoxia and is often increased in the vicinity of necrosis areas [28–30]. VEGF also induces fenestrations in the endothelium of small veins and capillaries, even in tissues where microvascularisation

does not normally present fenestrations, which explains partially the increased permeability of tumoral vessels [31, 32].

It was suggested that *vascular microdensity (MVD)* could be an independent factor of prognosis and metastases. The relationship between intratumoral microvascular density and increased risk for tumoral recurrences and metastases was observed in the carcinomas of mammary glands, pulmonary carcinomas, and in melanomas [33–35]. More research on colorectal carcinomas has demonstrated the tight correlation between vascular microdensity and metastases in regional lymph nodes and in distant sites [36, 37] (Figures 1 and 2).

Determining vessel microdensity requires the use of immunohistochemical techniques for evidencing endothelial cells. The most used markers are the antigen correlated with the factor VIII, CD31, and CD34.

Recently, Itoh J *et al.* [38] have employed confocal microscopy to visualize three-dimensional angiogenesis, a laborious and expensive technique. The density of small vessels is assessed in areas that contain the most numerous small venules (microvessels) and capillaries. These hot vascular spots (“hot spots”) can have any location, but are encountered more frequently at the tumoral edge or in the front of tumoral invasion [9].

Studies have shown a tight correlation between the expression of VEGF and an increased density of tumoral microvascularisation (MVD), the degree of malignancy and metastasizing play an important role in the tumoral growth, infiltration, and metastasizing in gastric neoplasm [39]. Therefore, the expressions of VEGF and MVD have a prognostic significance. Heterogeneity of the expression of VEGF and of forming of new vessels was observed in the tumoral tissue, deficient in the basal membrane.

This aspect demonstrates the hyperpermeability of neovascularisation, which facilitates penetration of tumoral cells into blood vessels and metastasizing. The expression of VEGF manifested through positive cells located in the center of the tumor or at the periphery of necrosis areas can be explained by the presence of hypoxia, which may stimulate the biologic expression and activity of VEGF [40, 41].

The notion of the use of VEGF as an ideal target for blocking tumor angiogenesis was successfully confirmed in laboratory experiments [42–45]. When anti-VEGF antibodies were administered in doses of 200 µg/day, for 14 consecutive days, a greater rate of tumoral growth suppression was observed (76.2%) in mice inoculated with human gastric tumoral cells. In these experiments, suppression was continued for 3–5 days after cessation of treatment [45]. Further studies are required in the future in order to evaluate the possibility of combining VEGF antibodies with conventional oncological agents.

Our observations performed on 18 cases of gastric carcinoma have demonstrated VEGF expression in 11 cases, with cytoplasmatic and granular pattern (Figure 3).

We also noted a positive reaction in areas of severe dysplasia limitroph to the tumor.

☞ The expression of COX-2 in GC

COX represents a key enzyme involved in the conversion of arachidonic acid in prostaglandins, two isoforms being identified, COX-1 and COX-2. COX-1 is expressed in numerous tissues and is considered to be involved in various physiological functions, while COX-2 is induced by pathogenic stimuli such as inflammation, various growth factors and cytokines produced by tumoral cells [46]. Numerous studies were performed in view of determining the relationship between COX-2 and tumoral angiogenesis, as well as the development and progression of gastric cancer.

Normally, gastric mucosa in humans does not express detectable levels of COX-2 protein. A study performed in China [47] on resection samples in 45 patients with gastric adenocarcinoma, has shown an immunohistochemical expression rate of COX-2 significantly greater in gastric cancer in comparison with peritumoral tissues. Also, the expression of COX-2 was proved as cytoplasmatic immune coloration not only in cancerous cells but also in precancerous lesions such as atypical hyperplasia and intestinal metaplasia [48–50]. These data demonstrate overexpressing of COX-2 in gastric cancer in humans, suggesting the fact that the enzyme COX-2 could play an important role in the occurrence of gastric cancer, being a relatively early event in gastric carcinogenesis [47].

The expression of COX-2 in gastric adenocarcinoma is greater in comparison with peritumoral tissues and is correlated with lymph node metastasizing and depth of invasion, suggesting that COX-2 could also be associated with the occurrence and progression of gastric neoplasm [51, 52]. The expression of COX-2 was tightly correlated with microvascular density, indicating the possible involvement of COX-2 in tumoral angiogenesis in gastric cancer. It is possible that COX-2-induced angiogenesis could be one of the mechanisms through which this promotes the invasion and metastasizing in gastric cancer. For these reasons, COX-2 could represent a new anti-angiogenic therapeutic target.

Several studies have shown the prophylactic effect of NSAID on colorectal carcinoma and their therapeutic effects on colonic polyps [53]. It is considered that the mechanism of action of NSAID is to inhibit COX-2, possibly causing lethal side effects, reason for which they are not for long-term application in order to prevent tumor genesis.

Several researchers have verified the relationship between COX-2 and angiogenesis, as well as the effects of NSAID on cells of vascular endothelium [54]. It was observed that Sulindac and Celecoxib significantly reduce the quantity of blood vessels in xenographs and microvascular density [55, 56].

Inhibitors of COX-2 perform their antineoplastic effects through suppression of the antiapoptotic gene Bcl-2 and reducing the angiogenesis in gastric carcinoma, thus affecting the tumoral nourishment, inhibiting the proliferation and inducing apoptosis of gastric neoplastic cells [57].

Figure 1 – Normal colorectal mucosa. Small vessels, uniformly distributed in the chorion (CD34, DAB ×400)

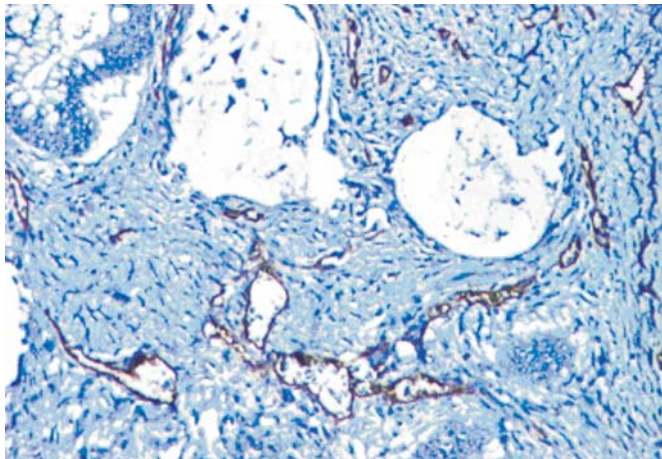
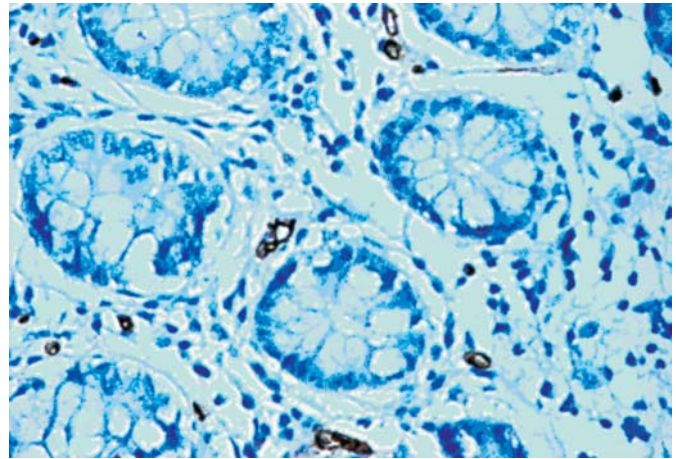


Figure 2 – Numerous unequal neovessels in the front of tumoral invasion (CD34, DAB ×100)

Figure 3 – Gastric adenocarcinoma with cytoplasmatic and granular immune staining pattern (VEGF, DAB ×400)

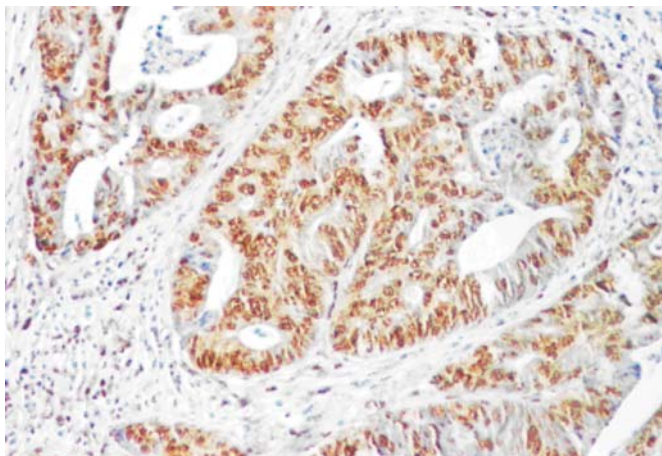
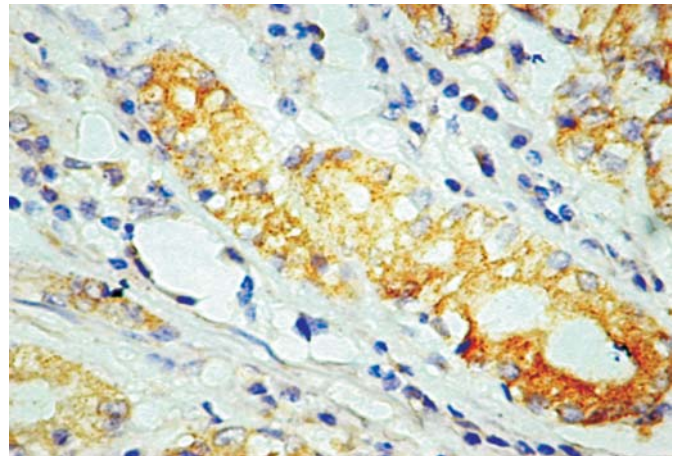


Figure 4 – p53 immune reaction in gastric adenocarcinoma (DAB, ×100)

CD44 represents an adhesion molecule on the cell surface, involved in intercellular adhesions as well as adhesions between cell and matrix. The expression of its 6 version has proved especially to be in close correlation with cell mobility, carcinogenesis, progression and metastasizing of gastric cancer. The influence of the COX-2 inhibitors on the expression of CD44v6 has been evaluated using animal models. The frequent presence of cells positive for CD44v6 at tumor periphery has been observed through immunohistochemistry, with a tendency to surround blood vessels. The positivism of immunocoloration was weaker in patients in whom COX-2 inhibitors were administered, which demonstrates that these medicines reduce the invasivity and capacity to metastasize of gastric neoplasm [58].

Therefore, in the case of gastric cancer, COX-2 inhibitors suppress cell proliferation, induce apoptosis, reduce angiogenesis and invasiveness [59]. Further studies are necessary to certify the effects of anti-inflammatories on the development of gastric adenocarcinoma.

☐ **The expression of inducible nitric oxide synthetase (iNOS), p53, and VEGF in gastric precancerous lesions and gastric cancer**

Inducible nitric oxide synthetase (iNOS) represents one of the isoforms of the nitric oxide synthetase which catalyzes the formation of nitric oxide, regulator of vascular permeability.

P53 suppresses angiogenesis through reduction of VEGF and iNOS. P53 enhances the induction of the VEGF expression by the C protein kinase [60].

Feng CW *et al.* [61] have studied the alterations of the expression of p53, iNOS, and VEGF in normal tissues and in tissues with lesions with various degrees of severity – superficial chronic gastritis, atrophic chronic gastritis, intestinal metaplasia, gastric dysplasia and cancer, with or without lymph nodes metastases, on resection samples from patients with gastric cancer and gastric biopsies taken from asymptomatic subjects.

The results obtained revealed the fact that immunoreactivity for p53, iNOS, and VEGF occurs in early stages of gastric carcinogenesis. Cells positive for immune staining presented invariably an activity of cellular proliferation. In parallel with the progression of lesions from normal to superficial chronic gastritis, then to atrophic chronic gastritis, intestinal metaplasia, dysplasia, and finally gastric cancer, the rates of immune staining also increased significantly for p53, iNOS, and VEGF, this growing tendency presenting a linear correlation with the progression of lesions. This data indicates that accumulation of the p53 protein and increased expression of iNOS and VEGF can represent important molecular events involved in early stages of gastric carcinogenesis [61].

An increased incidence of immune staining for p53 and iNOS (36%) was observed, as well as for p53 and VEGF (35%). Several studies have indicated that the p53 protein detected immunohistochemically represents

mostly the mutant p53. As the accumulation of the p53 protein was correlated with a mild differentiation and with the T2, T3, and T4 TNM stages of gastric cancer, it may be concluded that this accumulation could determine the loss of control on cellular proliferation and on inhibition of iNOS and VEGF, possibly due to mutations of the p53 gene.

For these reasons, the concomitantly increased expression of iNOS, VEGF, and accumulation of the p53 protein, can represent important events, which induce gastric carcinogenesis and are associated with an unfavorable clinical picture, and can be used as biomarkers for evaluating the risk for gastric cancer development.

Recent studies on Japanese patients with gastric cancer have revealed that the increased expression of VEGF and the accumulation of the p53 protein are associated with intratumoral angiogenesis [62] and with an unfavorable prognosis [63–65]. Rajnkova A *et al.* suggested that the increased expression of iNOS could initiate the progression towards gastric cancer by ensuring an advantage for selective growth of tumoral cells with non-functional p53 [66].

An increased incidence of overexpression of iNOS, VEGF, and accumulation of the p53 protein was reported in the case of gastric biopsies with intestinal metaplasia, which demonstrates that this lesion represents an unstable case in which active molecular modifications are present.

A correlation was observed between immune staining rates for iNOS and lymph node metastasizing of gastric cancer, and also between the increased expression of VEGF, accumulation of the p53 protein, and the TNM stage, as well as the degree of differentiation of gastric neoplasm, data which indicate a possible use as biomarkers of aggressiveness in GC (Figure 4).

☐ **The expression of PTEN in GC**

PTEN, tumor suppressor gene, contributes to cellular differentiation, reproduction, and apoptosis as well as to cell adhesion and mobility. Certain studies have shown a reduction in the expression of the PTEN encoded protein, due to genetic alterations such as mutations, loss of heterozygosity or hypermethylation, in GC, prostate and breast cancer [67, 68].

Low expression of the product of the PTEN gene was correlated with the depth of invasion and the presence of metastases in GC, as well as with the growth pattern and degree of differentiation; the expression of PTEN is more increased in the solid, intestinal, well-differentiated type versus the diffuse, poorly differentiated type. These results suggest that deletion or reduction of the expression of the PTEN protein could facilitate invasion and metastasizing in GC, by reduction of cellular adhesion and stimulating the synthesis of metalloproteinases and VEGF, therefore of tumor angiogenesis. As for the relationship with tumor angiogenesis, an inverse proportionally relationship was observed between the expression of PTEN and MVD [69, 70].

Moreover, the poor expression of PTEN could in turn reduce the expression of Caspase-3, which determines an alteration of tumoral cell apoptosis, representing the molecular mechanism of PTEN contribution to tumor genesis and progression of gastric cancer [69, 71].

☞ **The expression of thrombospondin-1 (TSP-1) in GC**

TSP-1 plays a role in angiogenesis; however, presently, the contribution of TSP-1 to neovascularisation and tumoral progression is controversial. It was presumed that TSP-1 functions as a stimulator, respectively inhibitor of angiogenesis in a manner specific for each organ and according to the concentration (in low concentrations it inhibits angiogenesis, and in large concentrations it stimulates it).

An intense expression of TSP-1 was detected immunohistochemically in 38% of GC; a variably increased expression of TSP-1 mRNA in most lines of gastric neoplastic cells was revealed through RT-PCR analyses. A positive correlation was observed between the expression of TSP-1 and intratumoral MVD in GC.

Some studies suggested that the product of the wild gene p53 increases the expression of TSP-1 and reduced through mutations of p53; however, data is contradictory. Recently it was observed that the expression of TSP-1 is reduced through hypermethylation of the promoting region, in 30% of gastric neoplasms, regardless of the status of the gene p53.

Therefore, TSP-1 is intensely expressed in the cytoplasm of gastric carcinomas, being positively associated with neovascularisation. These aspects indicate a possible participation of TSP-1 to the angiogenic phenotype of GC [72].

☞ **Cellular biologic behavior and relationship with angiogenesis in intraepithelial gastric neoplasm (tubular gastric adenomas) and intramucous gastric cancer**

Numerous studies evaluated the correlation between apoptosis tumoral microvascularisation and the expression of thymidine-phosphorilase, representing an angiogenic factor in early and advanced gastric cancer.

A Japanese study compared gastric tubular adenomas (TAs) and gastric intramucous adenocarcinomas (IMACs) of intestinal type in regard to cellular proliferation (Ki67 immune histochemical determination), apoptosis (employing the TUNEL method), intratumoral microvascularisation (CD34 immune staining), and immunochemical expressions of thymidine-phosphorilase and p53.

Unlike TAs, intramucous adenocarcinomas presented a significantly higher proliferative activity, a lower apoptosis, and a significantly higher number of intratumoral microvessels. In other words, tubular adenomas demonstrated a more static nature in comparison with IMACs.

The study showed the presence of a significant inverse correlation between the apoptotic index and the density of tumoral microvascularisation, involving a tight relationship between the blood flow and the occurrence of apoptosis in tumoral cells. The expression of thymidine-phosphorilase was significantly correlated with MVD, which might suggest that this ensures an advantage for tumoral growth in the case of TAs and IMACs, through the formation of an intratumoral microenvironment rich in blood vessels [73].

Osaki M *et al.* [74] confirmed the existence of a significant correlation between the expression of thymidine-phosphorilase and raised MVD, which attenuated apoptosis in the case of incipient gastric carcinomas with invasion of the submucosa, as well as in advanced carcinomas, the apoptotic index being much more reduced in the case of the latter.

It was demonstrated that the mutation of the p53 gene or the expression of P53 is more frequent in gastric carcinomas as compared with TAs, this mutation reducing the cellular apoptosis in human GC *in vivo*.

☞ **The role of the mast cell infiltrate surrounding gastric neoplastic cells in the process of tumoral angiogenesis and metastasizing**

Accumulation of mast cells at the periphery of neoplastic areas was observed for a long time (initially by Westphal in 1981), the functional significance of this phenomenon representing, however, a controversial subject. An initial hypothesis refers to the possibility that the accumulation of mast cells should represent part of a host defense immunological reaction, as mastocytes proved to have a cytotoxic effect in the case of certain tumors (especially for those sensitive to TNF- α). However, this hypothesis does not explain the fact that tumors continue to progress despite the increased incidence of these cells of the immune system. For this reason, a second possibility was taken into consideration through which the products of mast cell degranulation could promote tumoral growth and metastasizing.

Several studies have demonstrated the presence of an increased number of mast cells, as an early and persistent aspect in a variety of solid tumors.

In order to identify the role of mast cells surrounding gastric tumoral cells, the relationship between the number of mastocytes and tumor microvascularisation was investigated, using special staining methods for mastocytes (toluidine blue) and immunohistochemical reactions (antibodies anti-factor VIII / CD34) for MVD. Numerous studies have detected an increased number of mast cells in GC.

Their number was correlated significantly with the depth of invasion, lymph node metastases, lymphatic or blood invasion, the degree of histological differentiation, as well as the number of blood vessels surrounding gastric tumoral cells. Mast cells, most of them degranulated, were localized in the vicinity of the areas of neovascularisation.

Accumulation of mast cells in tumors is probably due to the active migration of mast cells or mast cells

precursors in the tumor area, as a response to chemo-attractants derived from the tumor. The existence of the mast cell motility factor was proved, as well as the factor of autocrine motility factor, derived from the tumor, involved in this migration process.

Numerous mast cells associated to tumors undergo a degranulation process, releasing granular components such as heparin and histamines, as well as numerous polypeptidic growth factors, among which bFGF and VEGF, which enhance proliferation and migration of endothelial cells, and induce the expression of adhesion molecules on the surface of endothelial cells, thus determining an increase in angiogenesis and tumor metastasizing. Tumors injected in mast cell-deficient mice or in mice treated with inhibitors of mast cell degranulation, presented reduced vascularisation, growth, and metastasizing, indicating the fact that mastocyte granular products stimulate tumoral growth and metastasizing; this data comes to support the use of anti-mast cell agents in the setting of antineoplastic therapy [75–77].

☐ **The presence of micrometastases in the hematogenous marrow in GC and their relationship with tumoral angiogenesis**

Development of metastases in neoplastic patients after curative resection of the primary tumor represents a serious problem, as early stages in the forming of micrometastases are difficult to distinguish. Subclinical dissemination of tumoral cells can be detected by immunological staining of cells. Cytokeratins represent essential constituents of the cytoskeleton of epithelial cells, normal as well as malignant; for this reason, they can represent the markers of the epithelial origin of cells (78).

Second only to the liver, the skeleton represents the most frequent location of distant metastases resulting from GC. Schlimok *J et al.* [79] reported the presence of tumoral cells in the hematogenous marrow, detected through immunohistochemical evaluation of cytokeratin, in 53% of patients with GC at the time of primary tumor resection. The apparent discrepancy between clinically manifest bone metastases, which are rare, and micrometastases in the marrow frequently detected immunohistochemically, can be explained through the reduced proliferative behavior of cells and their latency status frequently invoked. Jauch *KW et al.* [80] concluded that positive marrow samples represent a surrogate marker for general dissemination of tumoral cells or minimal residual disease, rather than the beginning of metastatic growth in the skeletal system. The presence of disseminated cells in the hematogenous marrow could also indicate the fact that tumoral cells have reached the peritoneum, liver, or lung. Survival of the cytokeratin-positive patients is more reduced than that of cytokeratin-negative patients. It was observed that VEGF-positive tumors associated with increased vascular density often-present cytokeratin-positive cells in the hematogenous marrow, the occurrence of these micrometastases possibly being linked to angiogenesis

in the primary tumor. The presence of micrometastases was noted even in patients with intramucous gastric neoplasm; therefore, insemination with neoplastic cells can occur even in early tumoral stages.

Examining lymph nodes excised from patients with early gastric cancer apparent with no lymph node involvement, it was noted that only 23.5% of patients presented cytokeratin-positive cells at the time of primary tumor resection, which determined the classification of these patients in a more advanced TNM stage, with a more reserved prognosis. As this data possibly suggest the presence of metastases in the lymph nodes of patients with GC, the necessity to practice the prophylactic lymph node dissection is taken into consideration, in patients with GC in view of improving the survival rate, as well as, probably, in those with incipient GC, for increasing the curability.

Adjuvant chemotherapy and the use of antiangiogenic agents (TNP-470, anti-VEGF antibodies, and angiostatin) could also be efficient in preventing micrometastases of GC [78].

Particularities of antiangiogenic therapy

Numerous clinical trials have been performed so far on the effect of angiogenic inhibitors, as anti-VEGF antibodies, angiostatin and endostatin, inhibitors of metalloproteinases, TNP-740, and many others, in patients with metastasized neoplasms.

Antiangiogenic therapy is mainly directed towards small foci of cells in proliferation of the capillary endothelium of the tumoral bed or from metastatic sites. For this reason, inhibitors of angiogenesis in general do not suppress the hematogenous marrow; do not determine gastrointestinal symptomatology or hair loss.

As inhibitors of angiogenesis reduce neovascularisation through inhibition of the proliferation and migration of endothelial cells and not through a direct cytotoxic effect, it is necessary to administer them throughout a longer period in comparison with conventional cytotoxic agents.

Unlike conventional chemotherapy which requires pause periods which allow regeneration of normal cells in the marrow and gastrointestinal tract, antiangiogenic therapy is administered continuously, experimental studies revealing a cumulative effect; therefore, the longer the inhibitor is administered, the smaller the possibility for tumor recurrence after cessation of treatment.

The resistance phenomenon to angiogenic inhibitors did not represent a major problem in long-term experimental studies and in clinical trials. This therapy was proposed as a strategy to prevent the resistance gained to classical antitumoral agents.

A greater efficiency was observed on experimental models in the case of combining the antiangiogenic therapy with the cytotoxic one (curative effect) than for each of them taken separately (inhibition effect).

Moreover, antiangiogenic therapy could be used in combination with other treatment modalities, such as immune therapy or gene therapy. In conclusion, these inhibitors ensure a less toxic treatment and with a smaller risk for occurrence of resistance [81–84].

Future directions

Conventional antitumoral treatment includes numerous modalities, all with the purpose of directly targeting tumoral cells or preventing their proliferation. Tumoral population is not stable, manifesting, through its genetic, epigenetic and microenvironment heterogeneity, a wide array of expressions and behaviors of tumoral cells in continuous change. Based on these considerations, the therapy directed at tumoral vascularisation (i.e., at the population of endothelial cells of the microvascularisation in the tumoral bed) was taken into consideration; it is based on tumoral suppression subsequent to inhibition of associated vascularisation.

Researches in the field of angiogenesis expanded greatly recently. Therefore, inhibitors of angiogenesis are studied nowadays for their possible use in non-neoplastic conditions as well.

Presently there are a few fundamental questions: Can the debut of angiogenic activity be detected in the blood or other fluids? Can the angiogenic process be manipulated through genetic therapies? These are only a few of the questions that incite to continuing angiogenesis-related research.

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