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GASTRIC CANCER

Heparanase expression, degradation of basement membrane and low degree of infiltration by immunocytes correlate with invasion and progression of human gastric cancer

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Abstract

AIM: To disclose the mechanisms that accelerate or limit tumor invasion and metastasis in gastric cancer patients.

METHODS: The heparanase expression, continuity of basement, degree of infiltration by dendritic cells and lymphocytes in gastric cancer tissues from 33 the early and late stage patients were examined by immunohistochemistry, *in situ* hybridization and transmission electron microscopy.

RESULTS: Heparanase mRNA expression in the late stage patients with gastric cancer was stronger than that in the early stage gastric cancer patients. In the early stage gastric cancer tissues, basement membrane (BM) appeared intact, whereas in the late stage, discontinuous BM was often present. The density of S100 protein positive tumor infiltrating dendritic cells (TIDC) in the early stage gastric cancer tissues was higher than that in the late stage. The infiltrating degree of tumor infiltrating lymphocytes (TIL) in the early stage patients whose tumor tissues contained a high density of TIDC was significantly higher than that in the late stage gastric cancer tissues patients with a low density of TIDC. There were few cancer cells penetrated through the continuous BM of cancer nests in the early stage gastric cancers, but many cancer cells were found outside of the defective BM of cancer nests in the late stage.

CONCLUSION: Our results suggest that strong heparanase expression is related with the degradation of BM which allows or accelerates tumor invasion and metastasis. However, high density of TIDC and degree of infiltration by TIL are associated with tumor progression in human gastric cancers.

Key words: Heparanase; Basement membrane; Tumor infiltrating dendritic cell; Tumor infiltrating lymphocyte; Gastric cancer

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INTRODUCTION

Gastric cancer is one of the most aggressive malignant tumors, and its incidence is higher than that of any other gastrointestinal malignancy. The prognosis of patients with gastric cancer is often poor, due to tumor invasion and metastasis which are the most common causes of death in gastric cancer[1,2].

Degradation of basement membrane (BM) and extracellular matrix (ECM) around tumor is considered to be associated with invasion and metastasis of gastric cancer[3]. Heparanase is an endo-β-D-glucuronidase that specifically cleaves carbohydrate chain of heparan sulfate proteoglycans (HSPG)[4]. HSPGs are the main component of extracellular matrix and basement membrane which play a barrier to prevent tumor cells from invasion and metastasis[5]. Previous studies

have shown that heparanase, produced by malignant tumor cells, mediates degradation of heparan sulfate proteoglycans in the extracellular matrix and basement membrane around tumors, and their expression correlateds with the degree of tumor invasion and metastasis in several human malignant tumors[6-8].

Progression of malignant tumors is also restricted by host defense mechanisms[9,10]. The tumor infiltrating dendritic cells and lymphocytes are chief immunocytes that inhibite malignant tumor cells from invasion and metastasis. Many authors reported that the infiltration grade of tumor infiltrating dendritic cells are associated with patient survival and prognosis in a large variety of human malignancies[10-15]. Recent studies have shown that the number of tumor infiltrating lymphocytes is correlated with the progression of human carcinoma[16-18]. However, to our knowledge, the correlation between heparanase expression and infiltration degree of tumor infiltrating dendritic cells and lymphocytes has not been reported so far. The present study was, therefore, undertaken to clarify the relationships between heparanase mRNA expression, degree of degradation of basement membrane, density of tumor infiltrating dendritic cells, infiltrating degree of tumor infiltrating lymphocytes, and tumor invasion and progression in human gastric cancer patients.

MATERIALS AND METHODS

Tumor samples

Tissue samples were obtained from 33 patients with primary gastric cancer who underwent curative surgery in the Second Clinical Hospital of Harbin Medical University (Harbin, China). Ten patients had stage Ⅰ, 8 stage Ⅱ, 7 stage Ⅲ, 8 stage Ⅳ cancer according to the TNM classification (UICC, TNM classification, 5th Edition, 1997)[19]. Histological stage grouping was evaluated. Stages Ⅰ and Ⅱ (n = 18) were referred to the early stage, stages Ⅲ and Ⅳ (n = 15) were referred to the late stage. All fresh tumor tissues were divided into two parts, one part was fixed in 0.1 mol/L phosphate buffer (pH 7.4) containing 4% paraformaldehyde for immunohistochemistry and *in situ* hybridization, the other part was immersed in 0.1 mol/L phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde for transmission electron microscopy.

In situ hybridization

Paraffin-embedded tissue sections were prepared for heparanase staining. Following reagents were purchased from Maxim Biotech (South San Francisco, CA, USA). Tissue sections (4 μm) were deparaffinized, dehydrated and incubated in 0.2 mol/L HCl for 20 min. After washed with 2 × SSC, the sections were incubated with proteinase K for 10 min at 37℃, fixed with PBS containing 4% paraformaldehyde for 5 min, washed with 2 × SSC, and then prehybridized for 2 h at 63℃ in a buffer containing 50% deionized formamide, 4 × SSC, 2 × Denhardt's solution and 250 μg/mL RNA. Hybridization was performed in 50% deionized formamide, 4 × SSC, 2 × Denhardt's solution, 10% dextran sulfate and 500 μg/mL RNA. The final concentration of DIG-labeled heparanase probe was about 500 ng/mL. The probe was placed on the section, covered with parafilm and incubated at 63℃ overnight in a moisture chamber. After hybridization, excess probes were removed by washing in 2 × SSC followed by RNase treatment with 100 U/mL RNase T1 at 37℃ for 30 min. The sections were washed at 65℃ in 2 × SSC for 10 min, washed three times in 0.2 × SSC and 50% deionized formamide (10 min each time), and incubated with an anti-DIG antibody conjugated with alkaline phosphatase. For the following color reaction, 5-bromo-4-chloro-3-indolyl phosphatase was used. Finally, the sections were counterstained with Mayer's hematoxylin.

Immunohistochemistry

Paraffin-embedded specimens were prepared for S100 protein immunohistochemical staining. The specimens were cut into 5-μm thick sections and mounted on glass slides. The sections were then deparaffinized in xylene for 20 min and dehydrated in ascending concentrations of ethanol. Endogenous peroxidase was blocked by incubating the sections with 3.0% H_2O_2 in methanol. After incubated in normal bovine serum for 10 min, the sections were incubated with anti-S100 protein antibody (Sigma, St. Louis, MO, USA) for 2 h at 37℃. After washed with PBS, the sections were incubated with biotinylated immunoglobulin and streptavidin conjugated to horseradish peroxidase (ABC kit, Sigma, St. Louis, MO, USA). Immunostaining was developed using DAB/ H_2O_2 solution. Finally, the sections were lightly counterstained with hematoxylin.

Transmission electron microscopy

Specimens fixed in 0.1 mol/L phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde were rinsed with the phosphate buffer and postfixed in 0.1 mol/L phosphate buffer containing 1% OsO_4 for 2 h, dehydrated through ascending concentrations of ethanol, and embedded in Epon 812 using aclar film (Nisshin EM, Tokyo, Japan). Semi-thin sections were stained with toluidine blue and observed under a light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a H-600 transmission electron microscope (Hitachi, Tokyo, Japan).

Statistical analysis

Under the light microscope, S100 protein immunohistochemistry and heparanase mRNA stained sections were examined using the image analysis system computer software (Beihan Image Centre, Beijing, China). Twenty sections from each kind of staining were analyzed, two high-power fields (× 400) (each field is 0.255 mm^2) were randomly selected from each section. The number and area density of positive cells in each section were automatically calculated by the computer. The results were expressed as mean ± SD. Student's t-test was used to compare the S100 protein positive cells and heparanase expressing cells in the early stage gastric

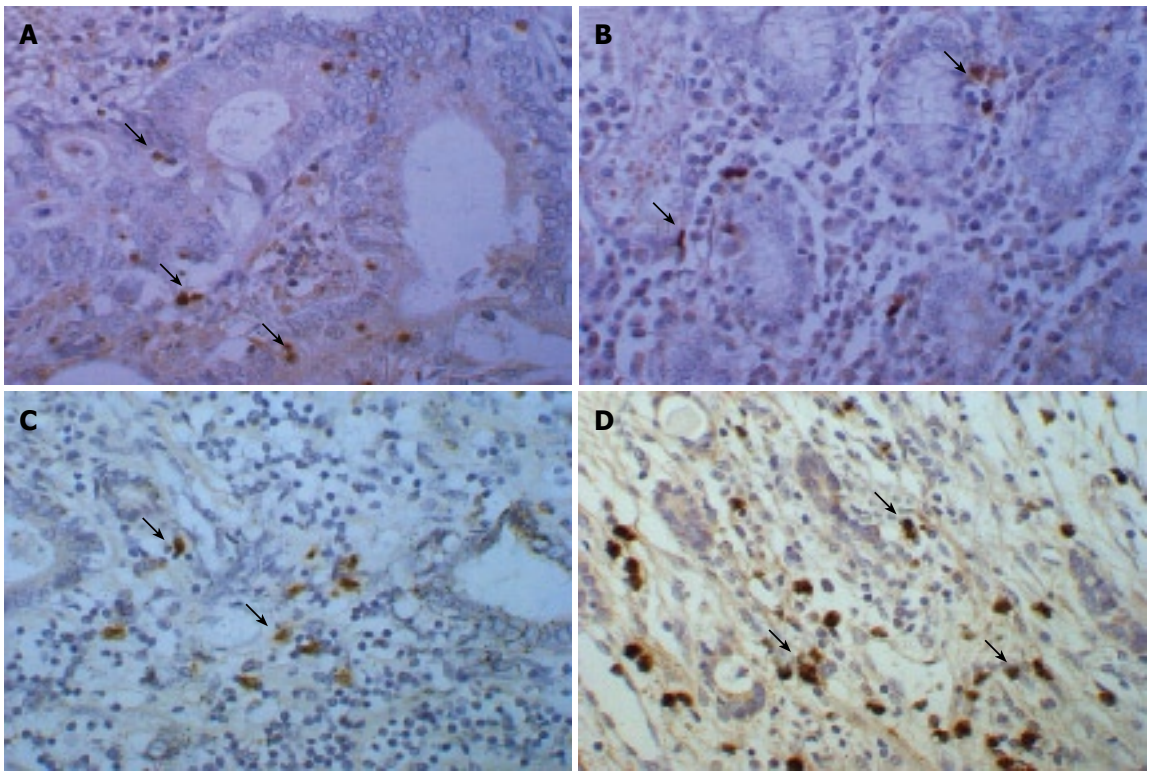

Figure 1 Expression and distribution of S100 protein and heparanase mRNA in gastric cancer tissues. Immunohistochemical staining of S100 protein (× 400) with a high density of tumor infiltrating dendritic cells positively stained for S100 protein in the early stage gastric cancer tissues (**A**) and a low density of such cells in the late stage gastric cancer tissues (**B**), heparanase mRNA expression by in situ hybridization (× 400) with a low heparanase mRNA expression level in the early stage gastric cancer tissues (**C**) and a high heparanase mRNA expression level in the late stage gastric cancer tissues (**D**) (Arrows: Positively expressed cells).

Table 1 Densities of S100 positive TIDC and heparanase expression in early and late gastric cancer tissues (mean ± SD)

	Densities of S100 positive TIDC			Heparanase expression		
	Early stage	Late stage	*P*-value	Early stage	Late stage	*P*-value
Cases (*n*)	18	15		18	15	
Number density	0.25 ± 0.19	0.03 ± 0.02	< 0.01	0.09 ± 0.08	0.33 ± 0.25	< 0.01
Area density	1.47 ± 1.15	0.21 ± 0.18	< 0.01	0.76 ± 0.64	3.47 ± 3.17	< 0.01

cancer tissues with those in the late stage gastric cancer tissues. $P < 0.05$ was considered statistically significant.

RESULTS

Distribution and density of tumor infiltrating dendritic cells in gastric cancer tissue

S100 protein positive cells showing typical morphology of dendritic cells and distinct cytoplasmic processes or veils, were detected in tissues from patients with gastric cancer at the early or late stage (Figure 1A and B). S100 protein positive cells were found mainly in stroma around the nests of cancer cells and connective tissue surrounding the tumor. In addition, S100 protein positive tumor infiltrating dendritic cells were also scattered among the cancer cells. Patients with gastric cancer at the early stage showed a high density of S100 protein positive tumor infiltrating dendritic cells (Figure 1A), while those at the late stage had a low density of S100 protein positive tumor infiltrating dendritic cells (Figure 1B). There was a significant difference in the density of S100 protein positive tumor infiltrating dendritic cells between gastric cancer patients at the early and late stage ($P < 0.01$, Table 1). The density of tumor infiltrating dendritic cells was significantly correlated with tumor invasion and clinical stage.

Heparanase mRNA expression in gastric cancer tissue

Heparanase mRNA positive labeling occurred mainly in cytoplasm and nuclei of gastric cancer cells. Heparanase mRNA was weakly expressed in the early stage gastric cancer tissues (Figure 1C) and strongly expressed in the late stage gastric cancer tissues (Figure 1D). The density of heparanase mRNA positive cells was significantly higher in the late stage gastric cancer tissues than in the early stage gastric cancer tissues ($P < 0.01$, Table 1). Heparanase mRNA expression was significantly correlated with invasion and TNM stage of gastric cancer.

Transmission electron microscopy (TEM)

The basement membrane was intact in the early stage

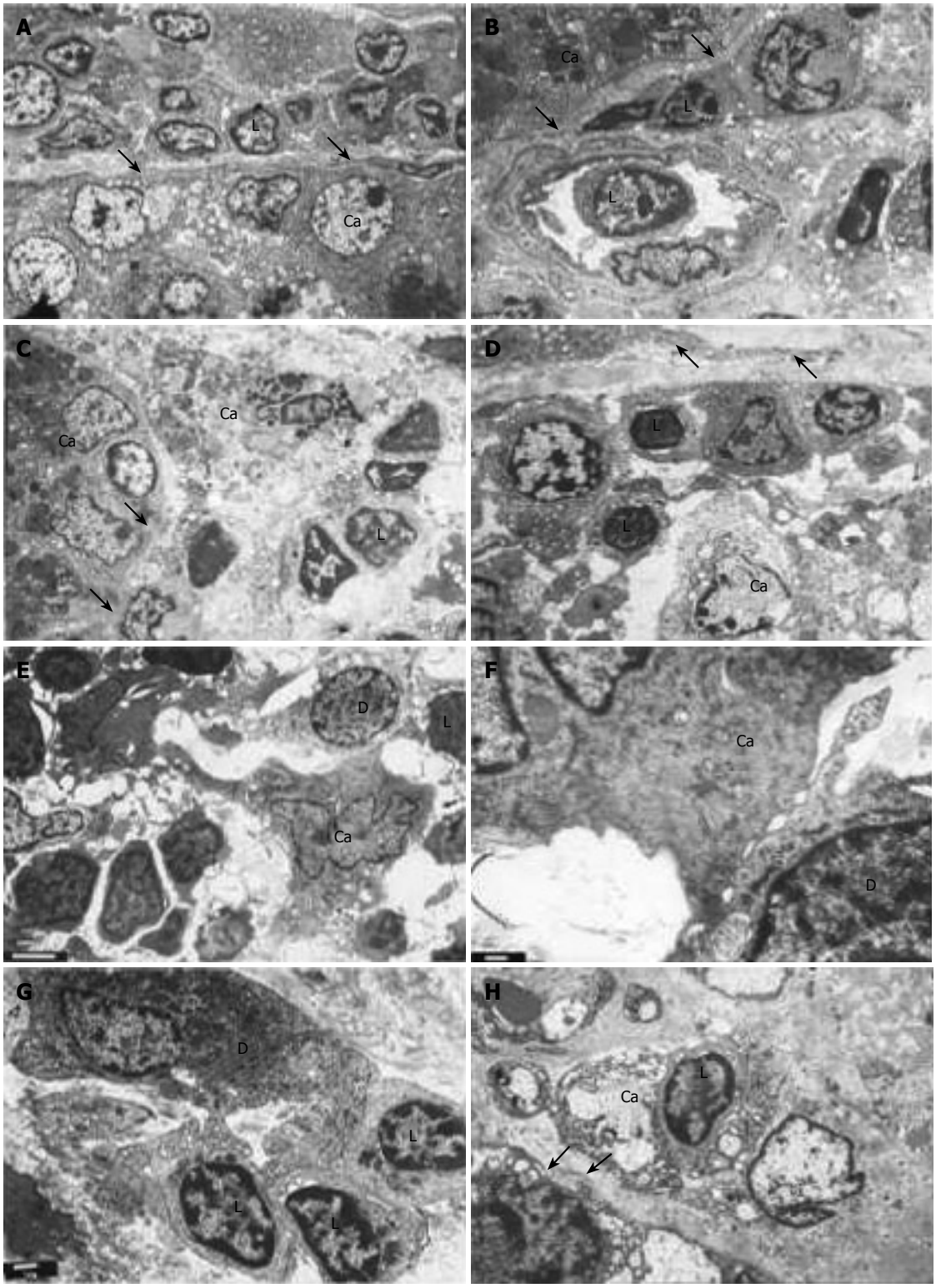

Figure 2 Transmission electron microscopy micrographs of the human gastric cancer tissues. (**A**)-(**D**) showing the early stage cancer tissues (Arrows indicate basement membrane). **A**: The continuous basement membrane which consisted of the electron-dense outer layer and the electron-lucent inner layer was observed. The numerous tumor infiltrating lymphocytes (L) were located in one side of the basement membrane (× 2500); **B** and **C**: The intact basement membrane was found on the margin of cancer nests. The tumor infiltrating lymphocytes (L) appeared around the cancer cell (Ca) (B × 4000; C × 3000); **D**: The cancer cell (Ca) was surrounded by the tumor infiltrating lymphocytes (L), and the basement membrane is clearly visualized (× 2500); (**E**)-(**H**) showing the late stage cancer tissues. **E**: The relationships were displayed between cancer cells (Ca) or tumor infiltrating lymphocytes (L) and tumor infiltrating dendritic cells (D). Note the tumor with absent basement membrane (× 4000); **F**: A higher magnification of **E** exhibited the contact relationship between the cancer cell (Ca) and tumor infiltrating dendritic cell (D) (× 20 000). **G**: The tumor infiltrating dendritic cell (D) was surrounded by several tumor infiltrating lymphocytes (L), and formed the dendritic cell-lymphocyte cluster (× 5000); **H**: The tumor infiltrating lymphocyte (L) appeared near the cancer cell (Ca), and the discontinuous or defective basement membrane of cancer nest can also be seen (double arrow) (× 5000).

gastric cancer tissue (Figure 2A-D). The continuous and well-formed basement membrane was found at the margin of

cancer nests in the early stage gastric cancer tissue (Figure 2B and C). The basement membrane was consisted of an electron-dense outer layer and an electron-lucent inner layer (Figure 2A and D). In contrast, the basement membrane at the rim of cancer nests was discontinuous and defective or absent in the late stage gastric cancer tissue (Figure 2E and H). Numerous tumor infiltrating lymphocytes were observed in the surrounding tissues of cancer nests and cells of the early stage gastric cancer (Figure 2B, C and D). In addition, many tumor infiltrating lymphocytes were arranged along one side of the basement membrane (Figure 2A). Lymphocytes in blood vessels were found near the cancer nests (Figure 2B), and invasion of cancer cells was noted outside of cancer nests (Figure 2C). The infiltrating degree of tumor infiltrating lymphocytes was high in the early stage gastric cancer tissues with intact basement membrane and few lymphocytes infiltrated into the tumor-surrounding tissue of the late stage gastric cancer with discontinuous basement membrane. Few cancer cells penetrated through the intact basement membrane of cancer nests in the early stage gastric cancer tissues, but many cancer cells were observed outside of the discontinuous basement membrane of cancer nests in the late stage gastric cancer tissues. There was a close contact between cancer cells or tumor infiltrating lymphocytes and tumor infiltrating dendritic cells (Figure 2E), and the contact between cancer cells and tumor infiltrating dendritic cells was also observed (Figure 2F). Many tumor infiltrating lymphocytes were distributed around the tumor infiltrating dendritic cells, forming a dendritic cell-lymphocyte cluster. Tumor infiltrating dendritic cells were closely contacted with tumor infiltrating lymphocytes (Figure 2G). Tumor infiltrating lymphocytes were also found near the cancer cells (Figure 2H).

DISCUSSION

It is generally accepted that one principal reason for the poor prognosis of patients with malignant tumors is the invasion and metastasis of cancer cells. The basement membrane plays an important role as a barrier in preventing cancer cells from invasion and metastasis[9]. The previous studies have demonstrated that heparanase which can degrade the basement membrane is one of the key enzymes involved in the tumor invasion and metastasis *in vivo*, such as pancreatic cancer, head and neck cancers, esophageal cancer, gastric cancer and colon cancer[7,8,20-22]. The heparanase can also shows *in vitro* human squamous cell carcinoma cell lines[23]. In the present study, we examined heparanase mRNA expression in the early and late stages of human gastric cancer by *in situ* hybridization. Our results show that heparanase mRNA expression was significantly higher in the late stage than in the early stage gastric cancer tissues ($P < 0.01$), and that heparanase mRNA expression was correlated significantly with tumor invasion and TNM stages of cancer.

Whether high expression of heparanase can promote the invasion of cancer cells by degrading the basement membrane remains unclear. Our TEM study has showed that intact basement membrane of cancer nests appeared in regions where heparanase expression was low in the early stage gastric cancer tissues, whereas discontinuous basement membrane of cancer nests was often present in places where heparanase expression was high in the late stage gastric cancer tissues. Our morphological observation directly proved that heparanase activity was associated with degradation of basement membrane. Lipponen[9] reported that invasion of superficial bladder cancer is related to the loss of continuous basement membrane, which is in agreement with our present TEM study.

In the present study, few cancer cells were observed to penetrate through the continuous basement membrane of cancer nests in the early stage gastric cancer tissue, but many cancer cells could be found outside of discontinuous basement membrane of cancer nests in the late stage gastric cancer tissue. These results suggest that the state of basement membrane, which is determined by heparanase mRNA levels, correlates with invasion of cancer cells. The present study also suggested that the discontinuity of basement membrane facilitate the invasion of cancer cells[23]. The discontinuity of basement membrane could probably results from the degradation of basement membrane by proteolytic enzymes, such as heparanase, which are presumably actively secreted by cancer cells[24].

The present study also showed that the state of basement membrane as a mechanical barrier and host immune defense system were interrelated. The immune defense system plays a critical role in preventing and limiting the development of malignant tumors[24]. The tumor infiltrating immunocytes situated around the tumor were considered a key factor for maintaining the status of local antitumor immunity[25]. The tumor infiltrating dendritic cells and lymphocytes are the main components of immunocytes in the tumor-surrounding tissues. Reportedly, the quantity of tumor infiltrating dendritic cells correlates with the clinical outcome of different tumor types[11,12,14,15]. Zeid and Muller demonstrated that the density of S100 positive dendritic cells in lung tumors is related to tumor subtype and differentiation, and a high dendritic cell density is associated with enhanced patient survival[11]. S100 protein has been widely used as a marker for identification of dendritic cells[26-28]. Tsujitani *et al* showed that the infiltration of dendritic cells is related to tumor invasion and lymph node metastasis in human gastric cancer[12]. A high number of dendritic cells in tumor tissue correlate with a good prognosis[29]. The present study showed that the density of S100 protein positive cells was higher in the early stage than in the late stage gastric cancer tissue ($P < 0.01$), suggesting that the density of tumor infiltrating dendritic cells correlates significantly with tumor invasion and clinical stages.

It was reported that invasion and metastasis of malignant tumor are related with the infiltrating degree of tumor infiltrating lymphocytes in tumor tissues[30]. Ropponen *et al* have shown the relationship between the number of tumor infiltrating lymphocytes and the prognosis of patients with colorectal cancer[31]. Aaltomaa *et al* demonstrated that a high number of

tumor infiltrating lymphocytes in tumor tissue correlate with a good prognosis of patients with breast cancer[32]. In the present study, the infiltrating degree of tumor infiltrating lymphocytes in the early stage gastric cancer patients whose tumor tissues contained a high density of tumor infiltrating dendritic cells was significantly higher than that in the late stage gastric cancer patients with a low density of these cells, indicating that the infiltrating degree of tumor infiltrating lymphocytes is associated with the progression of gastric cancer. These results suggest that there exists a certain relation between tumor infiltrating dendritic cells and lymphocytes. When the tumor becomes large, dendric cells and lymphocytes in the whole body are overwhelmed by a large number of tumor cells. In addition, these increased tumor cells will also prevent and limit infiltration by dendritic cells and lymphocytes. Thus, the decreases in tumor-infiltrating DCs and TILs may not be due to tumor development, but due to tumor growth.

Suzuki *et al* showed that dendritic cells are attached to groups of lymphocytes and form dendritic cell-lymphocyte clusters to promote T-cell activation for the generation of tumor-specific immunity in the invasive margin of the colorectal cancer stroma[33]. Dendritic cells present antigen to lymphocytes, stimulate naïve T lymphocyte proliferation and activation to kill tumor cells[34]. Bell *et al*[35] suggested that the peritumoral clustering of mature dendritic cells reflects a state in which they interact directly with clusters of tumor infiltrating lymphocytes to generate an antitumor immune response. In the present study, tumor infiltrating lymphocytes were distributed around tumor infiltrating dendritic cells and formed dendritic cell-lymphocyte cluster, and the close contacts were found between tumor infiltrating dendritic cell and tumor infiltrating lymphocytes. The contact between tumor infiltrating dendritic cells and lymphocytes may indicate the process that tumor infiltrating dendritic cells present antigen to lymphocytes to activate them for antitumor immunity[36,37].

Loss of integrity in basement membrane results from high heparanase expression. In the present study, few cancer cells were observed to penetrate through the continuous basement membrane of cancer nests in the early stage gastric cancer tissue, but many cancer cells could be found outside of the discontinuous basement membrane of cancer nests in the late stage gastric cancer tissue. These results suggest that the state of basement membrane, which is determined by heparanase, correlates with invasion of cancer cells. In the late stage gastric cancer tissue, increased cancer cells outside of discontinuous basement membrane of cancer nests could prevent and limit infiltration by dendritic cells and lymphocytes. Thus, heparanase expression or loss of integrity in basement membrane is associated with the infiltrating degree of tumor infiltrating dendritic cells and lymphocytes.

In summary, heparanase expression, degradation of basement membrane, density of tumor infiltrating dendritic cells and infiltrating degree of tumor infiltrating lymphocytes are associated with tumor invasion, TNM stages and progression in human gastric cancer. When the tumor has reached an advanced stage, discontinuous basement membrane, degraded by high expression heparanase, allows cancer cells to penetrate and is favorable to tumor invasion and metastasis, and can predict a poor prognosis of patients with gastric cancer. Moreover, at the late stage, a low degree of infiltration by dendritic cells and lymphocytes reflecting the presence of a weak local antitumor immune response in gastric cancer tissues also indicates that patients with less infiltrating immunocytes gastric cancer would have a poor prognosis, whereas the result is contrary in the early gastric cancer tissue. These factors including interactions between heparanase and basement membrane as well as between tumor infiltrating dendritic cells and lymphocytes may play a crucial role in tumor invasion and metastasis. Further study is required to understand the precise mechanism of interactions between both of them in the process of tumor invasion and metastasis.

COMMENTS

Background

The prognosis of patients with gastric cancer is often poor, due to tumor invasion and metastasis which are the most common causes for death of gastric cancer patients. It is crucially important to disclose the mechanisms underlying tumor invasion and metastasis. Heparanase, tumor infiltrating dendritic cells (TIDC) and tumor infiltrating lymphocytes (TIL) play a critical role in preventing and limiting the development of malignant tumors.

Research frontiers

Recent investigations have shown that heparanase expression which can degrade the basement membrane is one of the key enzymes involved in tumor invasion and metastasis, but few studies are available on the relationship between heparanase expression, basement membrane degradation, density of dendritic cells, infiltrating degree of lymphocytes, and tumor invasion and progression in gastric cancer patients.

Innovations and breakthroughs

High heparanase expression levels are related with the degradation of basement membrane which allows or accelerates tumor invasion and metastasis. However, high TIDC density and TIL infiltration degree are associated with progression of gastric cancer.

Applications

The study defined the mechanisms underlying tumor invasion and metastasis. Heparanase expression, basement membrane degradation, and TIDC and TIL infiltration degree, can be used as prognostic biomarkers for gastric cancer.

Terminology

Heparanase is an endo-β-D-glucuronidase that specifically cleaves the carbohydrate chain of heparan sulfate proteoglycan (HSPG). HSPG is the main component of extracellular matrix and basement membrane, which as a barrier can protect tumor cells from invasion and metastasis.

Peer review

This is a histopathological analysis of heparanase expression, dendric cells and lymphocytes infiltrating to tumor in patients gastric cancer at the early or late stage. In addition, the authors showed the electron microscopic pictures of gastric cancer tissue infiltrated by dendric cells and lymphocytes. This is an interesting report on heparanase expression in human gastric cancer.

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