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Homeostatic Mass Control in Gastric Non-Neoplastic Epithelia under Infection of *Helicobacter pylori*: An Immunohistochemical Analysis of Cell Growth, Stem Cells and Programmed Cell Death

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We evaluated homeostatic mass control in non-neoplastic gastric epithelia under *Helicobacter pylori* (HP) infection in the macroscopically normal-appearing mucosa resected from the stomach with gastric cancer, immunohistochemically analyzing the proliferation, kinetics of stem cells and programmed cell death occurring in them. Ki67 antigen-positive proliferating cells were found dominantly in the elongated neck portion, sparsely in the fundic areas and sporadically in the stroma with chronic infiltrates. CD117 could monitor the kinetics of gastric stem cells and showed its expression in two stages of gastric epithelial differentiation, namely, in transient cells from the gastric epithelial stem cells to the foveolar and glandular cells in the neck portion and in what are apparently progenitor cells from the gastric stem cells in the stroma among the infiltrates. Most of the nuclei were positive for ssDNA in the almost normal mucosa, suggesting DNA damage. Cleaved caspase-3-positive foveolar cells were noted under the surface, suggesting the suppression of apoptosis in the surface foveolar cells. Besides such apoptosis of the foveolar cells, in the severely inflamed mucosa apoptotic cells were found in the neck portion where most of the cells were Ki67 antigen-positive proliferating cells. Beclin-1 was recognized in the cytoplasm and in a few nuclei of the fundic glandular cells, suggesting their autophagic cell death and mutated beclin-1 in the nuclei. Taken together, the direct and indirect effects of HP infection on the gastric epithelial proliferation, differentiation and programmed cell death suggested the *in-situ* occurrence of gastric cancer under HP infection.

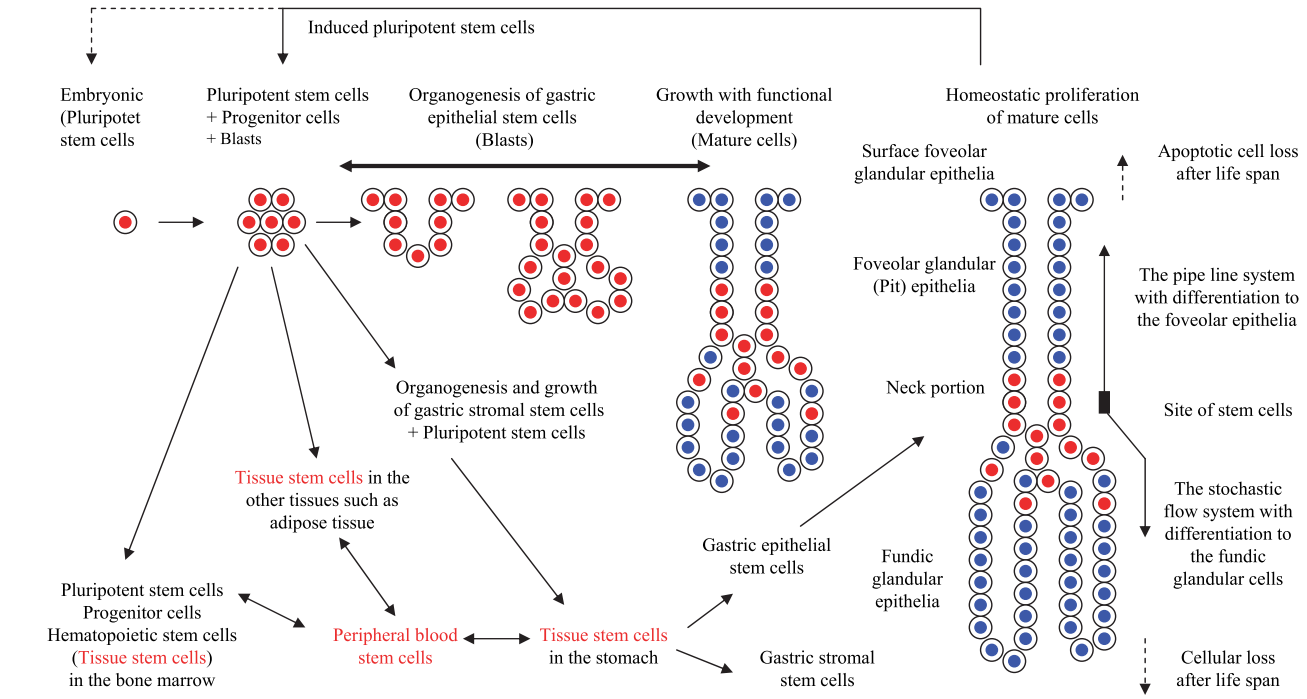
Key words: Helicobacter pylori, proliferation, stem cell, apoptosis, autophagy

I. Introduction

Helicobacter pylori (HP) causes inflammation, ulcer, adenocarcinoma and mucosa-associated lymphoid tissue (MALT) type lymphoma [2, 6, 30, 53]. However, it was recently reported that gastric cancer originates from bone

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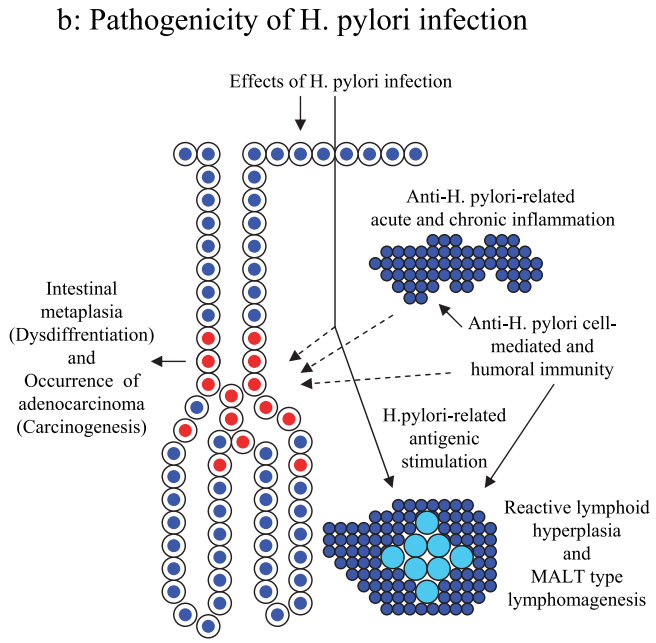


Fig. 1. Histogenesis of a human adult gastric fundic gland from viewpoints of the stem cells and the pathogenicity of HP infection. **a)** Conceptual relationship among stem cells in a gastric fundic gland in each developmental stage. Embryonic stem cells grow to become mature cells through progenitor cells and precursor cells (blasts). In stomach, mature cells reveal growth with functional development, maintaining a two-layered structure of the gastric fundic gland, and finally show homeostatic growth under homeostatic mass control in the adult. The primitive fundic gland is a simple tubular gland and comprises the blasts. After organogenesis the fetal fundic gland is a ramified tubular gland and comprises the mature cells. After birth the fundic glands continue the growth with functional development and reach to the stage of the adult gland with homeostatic growth. In the adult fundic glands, mature cells include gastric epithelial stem cells, proliferating mature cells and terminally differentiated cells. In the same manner, the other organs and tissues develop and some of them, such as bone marrow and adipose tissue, include their tissue (pluripotent) stem cells. In stomach, tissue stem cells (gastric stem cells) can differentiate to gastric epithelial stem cells. However, the relationship among these tissue (pluripotent) stem cells and the peripheral blood stem cells has yet to be elucidated. On the other hand, induced pluripotent stem cells from mature cells suggests the unique idea that stem cell phenotype can be acquired. Gastric fundic glandular cells begin to proliferate bidirectionally at the site of stem cells. In the pipe-line system, foveolar epithelial cells are differentiated and die after their terminal differentiation and their life span. In the stochastic flow system, fundic glandular cells are differentiated and die after their terminal differentiation and their life span. **b)** The pathogenicity of HP infection in gastric mucosa. HP infection directly and indirectly effects the homeostatic growth of the gastric mature cells and the gastric mucosal immunity. The effects on the latter contribute to persistent inflammation, appearance of reactive lymphoid hyperplasia and MALT-type lymphomagenesis. One of the effects on the former is explained to be due to CagA protein that suppresses apoptotic loss of surface foveolar epithelial cells and promotes persistent infection of HP. The effects on gastric epithelial stem cells in fundic glands have yet to be clearly elucidated.

marrow-derived stem cells [22, 23]. In order to understand the relationship between the HP-related carcinogenesis and the stem cell-related oncogenesis in the stomach, we set out to examine the renewal system of the gastric fundic glandular epithelia under HP infection from the viewpoint of the homeostatic mass control comprising the kinetics of stem cells in the stomach, their proliferation and their programmed cell death.

Embryonic (pluripotential) stem cells differentiate through progenitor cells, and precursor cells (blasts) into mature cells [26]. In the conceptual relationship among stem cells and mature cells in the organogenesis and the adult homeostatic growth of a gastric fundic gland (Fig. 1a), the gastric stem cells, a kind of tissue stem cell, would be the blasts, and the gastric epithelial stem cells would be the mature cells. It is reported that as gastric epithelial stem cells differentiate during gastric mucosal organogenesis they express MUC-1, MUC-2, MUC5AC and MUC-6 mucins [46]. In adults, pluripotential stem cells are located in the bone marrow, adipose tissue and stromal tissue [4], but the relationship among the pluripotential stem cells in these various tissues including peripheral blood stem cells has yet to be fully elucidated. It is now known that the progenitor cells have the plasticity to be pluripotential stem cells and can differentiate into all kinds of cells, depending on the microenvironment [4, 50]. It was recently reported that some stem cell-related genes will transform the induced mature cells to the pluripotential stem cells (induced pluripotent stem cells) [39]. In the adult homeostatic growth of the gastric fundic glands, probably depending on the microenvironment of the gastric mucosa, gastric stem cells must enter into the neck portion of the glands, become gastric epithelial stem cells and differentiate into the various kinds of gastric epithelia and endocrine cells. On the other hand, the renewal system (adult homeostatic growth) of the gastric fundic glands has been investigated from the viewpoints of proliferating cells [21], differentiation and programmed cell death [44] (Fig. 1a). It is shown that the gastric epithelial stem cells differentiate upward in the pipeline system to the foveolar epithelia and downward in the stochastic flow system to the fundic glandular epithelia [21]. The foveolar epithelia terminate in apoptosis on the surface of the gastric mucosa, but it is not clear how the fundic glandular epithelia terminate even though Stachura *et al.* [44] reported the ultrastructural morphology of their programmed cell death [5, 14]. Therefore, before a precise understanding of the renewal system of the gastric fundic glands can be achieved, more information is required regarding the supply of the stem cells and programmed cell death of the fundic glandular epithelia.

As for the pathogenicity of HP infection (Fig. 1b), CagA protein suppresses apoptosis of the surface foveolar epithelia after their life span [32]. Anti-HP natural and specific immunity yields acute and chronic inflammation with marked reactive lymphoid hyperplasia. The bacterial lipopolysaccharides (LPSs) may indirectly contribute to lymphomagenesis of MALT type lymphoma [16]. Dysimmunity against HP would result in modified histogenesis of MALT-type lymphoma [18]. As for intestinal metaplasia, altered gastric epithelial stem cells would differentiate into intestinal metaplastic cells [49]. However, the relationship in the molecular and genetic alterations between HP infection and gastric adenocarcinoma has yet to be elucidated.

From the viewpoint of multistep carcinogenesis [27], many molecular events such as mutations may have already accumulated in gastric stem cells by their entering into the gastric glands. But the molecular alterations in the final initiating step in carcinogenesis must be induced in the mature cells comprising gastric epithelial stem cells and their mature cells in the gastric glands. Therefore, it is important to see when and where the molecular alterations in the final initiating step in carcinogenesis occur in the gastric fundic glands under homeostatic mass control, and what the molecular alterations in the final initiating step in carcinogenesis are.

This study investigated non-neoplastic fundic glands in the resected stomach with cancer because carcinogenesis-related and not-apparent disorders would be elucidated more easily in them than in those in the resected stomach without cancer. This study then investigated the proliferation, the supply of the so-called stem cells and the programmed cell death in the gastric fundic glands under homeostatic mass control by means of the immunohistochemistry (IHC) of the proliferating cells, the stem cells, the scavenger macrophages, and the programmed cell death. As for the IHC of the stem cells, the adequate ordinary and supersensitive methods of IHC [17, 20] of the well-known anti-CD34, anti-CD117 and anti-CD133 antibodies were examined to label the stem cells in the ordinary fixed and paraffin-embedded bone marrow aspiration clot specimens and in the peripheral blood stem cells in peripheral blood tissue specimen (PBTS) [19] and then were applied to this investigation. As the IHC detecting programmed cell death comprising apoptosis, autophagic cell death and others [5, 14, 44] in normal-appearing gastric fundic mucosa, we used the ordinary and supersensitive IHC [17, 20] employing anti-cleaved caspase-3 and anti-beclin-1 antibodies [13, 29].

II. Materials and Methods

Ordinary processed and paraffin-embedded tissue sections of mostly normal-appearing gastric mucosal tissues specimens taken from the posterior wall (fundic glandular region) of 10 cases of the ordinary 10% formalin-fixed stomach resected because of gastric cancer. The clinicopathological data of these cases were not obtained.

On the hematoxylin and eosin (H&E)-stained specimens, the HP infestation and the chronic inflammation were evaluated according to the criteria of the Sydney system for gastroendoscopic biopsy specimens [7, 9, 33, 41].

Paraffin sections of three cases each of bone marrow aspiration clot specimens and peripheral blood tissue specimens (PBTS) [19] were employed to examine whether the IHC of CD34, CD117 and CD133 could label bone marrow-

tissue stem cells, hematopoietic stem cells and peripheral blood stem cells. The three patients of the bone marrow aspiration clot specimens were diagnosed pathologically as hyperplastic marrow without obvious diseases. The three patients of the PBTS were diagnosed hematologically as reactive leukocytosis without obvious diseases. None of these patients had gastric cancer.

This study was performed with the approval of the Kagoshima University Hospital Ethics Committee.

Immunohistochemistry (IHC)

1) Antibodies

Antibodies used were Ki67, CD34, CD117, CD133, muramidase, CD68, single-stranded DNA (ssDNA), cleaved caspase-3 and beclin-1, and are listed in Table 2. The antibody diluent solution was Antibody Diluent (ChemMate, S2022, Dako) in the case of the polymer method and 2% bovine serum albumin (BSA)-0.1% Tween 20-0.01 M phosphate buffer saline pH 7.2 (PBS) in the case of supersensitive IHC.

2) Antigen retrieval

Antigen retrieval (AR) was selected for each antibody as shown in Table 2. Heat-antigen retrieval (hAR) was performed, by incubating the sections in the solution and by heating for 5 min at 121°C using an autoclave, then cooling down to the room temperature, and finally rinsing the sections in PBS three times.

Enzymatic antigen retrieval (eAR) was performed by automatic staining using an autostainer (Dako), incubating the sections in the proteinase K solution for 10 min. This eAR was employed in the polymer method of ssDNA and in the supersensitive method of beclin-1.

3) Indirect enzyme-labeled method of IHC

The sections were deparaffinized by incubating sections in xylene for 10 min three times and in 100% ethanol for 10 min three times. Endogenous peroxidase in the sections was inactivated, by incubating the sections in 0.3% hydrogen peroxide methanol for 30 min. The sections were rinsed in PBS three times. Adequate AR for each antibody was performed as mentioned above and listed in Table 2.

The following procedures were performed using the autostainer. These procedures have been previously reported [17, 20]. Briefly, the ordinary sensitive method and the supersensitive method are described below. The after-reaction rinse solution was 0.1% Tween 20-0.05 M Tris-HCl buffer saline pH 7.6 solution (TBST) warmed to 35°C.

As the ordinary sensitive method, this study employed the polymer method that was performed as below. After blocking the non-specific binding of the primary antibody by incubating sections in 3% BSA-0.1% Tween 20-PBS for 15 min, the primary antibody reaction was performed for 1 hr (15 min for ssDNA). The reacted primary antibody was labeled by incubating section in the reagent solution (ChemMate Envision, Dako) of the polymer carrying secondary antibody and horse radish peroxidase (HRP) for 30 min and by 3'3-diaminobenzidine HCl (DAB)-hydrogen peroxide reaction (DAB+ liquid system, K3468, Dako) for 10 min. Nuclear counter-staining was made by incubating section in hematoxylin solution (ChemMate hematoxylin, S2020, Dako) for 2 min. After being removed from the autostainer, sections were dehydrated in 100% ethanol for 5 min three times, were passed through xylene, and were mounted in a plastic medium.

The supersensitive method employed in this study is based on the polymer method mentioned above [17, 20] and

Table 1. *Cases and epithelial lesions examined*

Case	Mucosa	Inflammation	Glandular lesion examined
A	Fundic (Fig. 2a)	Rare lymphocytes in thin interglandular stroma	Normal 1
B	Fundic	Rare lymphocytes in thin interglandular stroma	Normal 2
C	Fundic	Some lymphocytes in thin interglandular stroma	Inflamed 1
		Moderate infiltration of dominantly lymphocytes in parts in the upper portion of the mucosa. Tiny lymph follicles in the base portion of the mucosa.	Inflamed 2
D	Fundic	Moderate infiltration of dominantly lymphocytes in the upper portion of the mucosa. Tiny lymph follicle formation in the base portion of the mucosa.	Inflamed 3
E	Fundic (Fig. 2b)	Sever infiltration of dominantly lymphocytes in the upper portion of the mucosa. Marked lymph follicle formation in the lower and base portion of the mucosa.	Inflamed 4
F	Fundic	Sever infiltration of dominantly lymphocytes in the upper portion of the mucosa. Marked lymph follicle formation in the lower and base portion of the mucosa.	Inflamed 5
G	Metaplastic with residual fundic glands (Fig. 2c)	Moderate infiltration of dominantly lymphocytes in the upper and lower portion of the mucosa with focal completely intestinal metaplasia.	Residual 1
			Metaplastic 1
	Metaplastic	Some infiltration of dominantly lymphocytes in thin interglandular stroma in the mucosa with completely intestinal metaplasia.	Metaplastic 2

is different from the modified ImmunoMax method [19] and the DAKO CSA system, which comprise the streptavidin-biotin complex method and catalyzed reporter deposition (CARD) reaction. After the second inactivation of endogenous peroxidase by incubating sections in 3% hydrogen peroxide PBS solution for 5 min and the pretreatment blocking the non-specific binding of the primary antibody by incubating sections in 3% BSA-0.1% Tween 20-PBS for 15 min, the primary antibody reaction was performed for 15 min. The polymer reagent reaction was performed for 15 min with the pretreatment blocking the non-specific binding of the polymer reagent by incubating sections in 3% BSA-0.1% Tween 20-PBS for 15 min. Blocking the non-specific deposition of the biotinylated tyramide employed in the CARD reaction by incubating sections in 3% BSA-0.1% tween 20-PBS for 15 min, the CARD reaction employing biotinylated tyramide was made by incubating sections in the biotinylated tyramide reagent (Amplifying reagent, CSA system, K1500, Dako) for 15 min. The deposited biotinylated tyramide was visualized by streptavidin-HRP complex reagent reaction (Detection reagent, CSA system, K1500, Dako) for 15 min and DAB-hydrogen peroxide reaction (DAB+ liquid system, K3468, Dako) for 4 min. Nuclear counter-staining was made by incubating sections in hematoxylin solution (ChemMate hematoxylin, S2020, Dako) for 2 min. After being removed from the autostainer, sections were dehydrated in 100% ethanol for 5 min three times, were passed through xylene, and were mounted in a plastic medium.

In IHC, the combination of an adequate antigen retrieval for the antigen and a proper sensitive detection method employing the primary antibody against the antigen was performed (Table 1). The antigen detection by the IHC employing the primary antibody was described as the hAR/eAR-polymer/supersensitive method of the antigen in this article.

Microphotographs

Microphotographs of the specimens were taken using a microscope (BX-50, Olympus Co.) with a digital microscopic camera (Fuji HC-300). The exact length of the long axis of the microphotograph was 860 μm at ×10 magnification, 430 μm at ×20 magnification, and 215 μm at ×40 magnification.

III. Results

Histology of the sections examined

As shown in Table 1, the full length of the gastric fundic glands could be evaluated in 10 lesions from 7 specimens out of 10 cases.

The normal gastric fundic glandular mucosa (Table 1, Normal 1 and 2) showed rare lymphocytes in the thin interglandular stroma and HP were not seen, as shown in Figure 2a. According to the criteria of the Sydney system, the Normal 1 and 2 lesions were evaluated as follows: HP: normal; neutrophils: normal; mononuclear cells: normal; atrophy: normal; and intestinal metaplasia: normal.

Inflamed gastric glandular mucosa (Inflamed 1 to 5 in Table 1) showed hematoxylin-stained HP bodies in the surface mucous coat or in the glandular lumen in the foveolar portion. The inflammation could be categorized as mild, moderate and severe. The mildly inflamed mucosa showed some chronic inflammatory cells, dominantly lymphocytes, in the thin interglandular stroma (Table 1, Inflamed 1). The

Table 2. *Antibodies and antigen retrieval methods employed in this study*

Antibody			Antigen retrieval	
Name	Catalog No.	Dilution	Method	Solution
Ki67 antigen (MIB-1)	M7240, Dako	1:100	Autoclave	0.01 M citrate buffer pH 6
CD34 (QBEnd 10)	M7165, Dako	1:100	Autoclave	0.01 M citrate buffer pH 6
CD117	NCL-CD117, Novocastra Lab. Ltd.	1:40	Autoclave	EDTA solution
CD133	431133, clone 170411, Techne Co.	1:100	Autoclave	0.01 M citrate buffer pH 8
muramidase	A0099, Dako	1:500	Autoclave	0.01 M citrate buffer pH 6
CD68	PG-M1, M0876, Dako	1:100	Autoclave	0.01 M citrate buffer pH 6
single-stranded DNA (ssDNA)	A4506, Dako	1:100	Enzymatic treatment	200 μg/ml proteinase K
cleaved caspase-3	5A1, Asp175, Cell Signaling Co.	1:200	Autoclave	EDTA solution
beclin-1 (H-300)	Sc-11427, Santa Cruz Biotech. Inc.	1:50	Autoclave	0.01 M citrate buffer pH 8
			Enzymatic treatment	200 μg/ml proteinase K

Antibody-Dilution

The antibody dilution solution was Antibody Diluent (ChemMate, S2022, Dako) in the case of the polymer method and 2% Bovine serum albumin (BSA)-0.1% tween 20-0.01 M phosphate buffer saline pH 7.2 (PBS) in the case of the supersensitive immunohistochemistry.

Antigen retrieval-Method

Autoclave: Heated by means of autoclave at 121°C, for 5 min, and cooled to room temperature.

Enzymatic treatment: Treated with enzyme working solution in the autostainer for 10 min.

Antigen retrieval-Solution

0.01 M citrate buffer pH 6: S2031, ChemMate, Target Retrieval Solution, Dako, diluted with distilled water in 1:10.

EDTA solution: S3307, Antigen Retrieval Reagent, high pH, Dako, diluted with distilled water in 1:10.

0.01 M citrate buffer pH 8: 0.01 M citrate buffer pH 8 with 0.1% NP-40.

200 μg/ml proteinase K: 200 μg/ml proteinase K (Code No. 9033, Takara Bio Co.)-0.05 M Tris buffer saline pH 7.2.

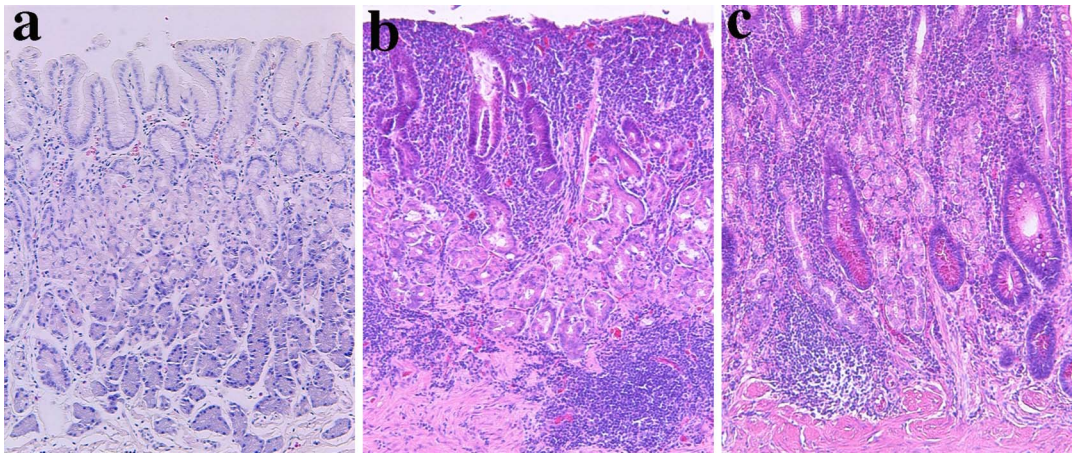


Fig. 2. Mostly normal-appeared mucosa taken from the resected stomach with cancer (H&E stain, ×10 magnification). **a)** Mostly normal gastric fundic mucosa. **b)** Inflamed gastric fundic mucosa with HP infection. Reactive lymphoid hyperplasia (developed mucosal associated lymphoid apparatus) is noted. **c)** Inflamed intestinal metaplastic gastric mucosa. There were obvious intestinal type glands with appearance of Paneth's cells.

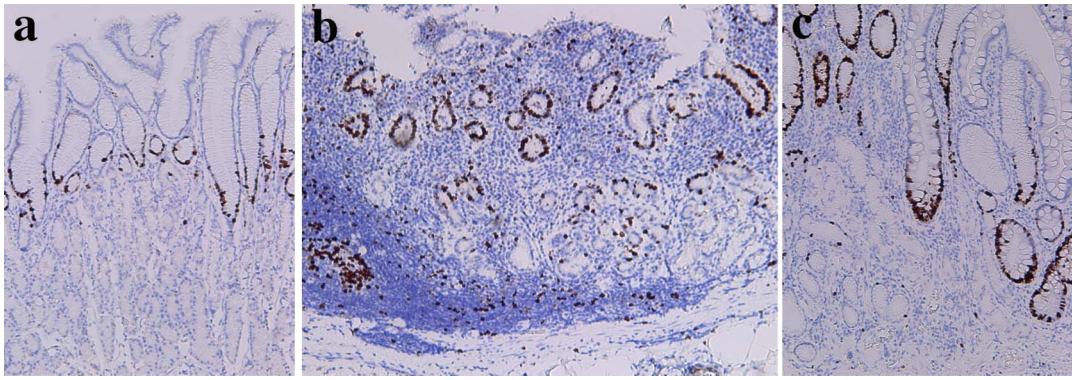


Fig. 3. Proliferating cells labeled by hAR-polymer method of Ki67 antigen (×10 magnification). **a)** Mostly normal gastric fundic mucosa. Some labeled proliferating cells located in the neck portion. **b)** Inflamed gastric mucosa with HP infection. Many labeled proliferating cells were seen in the elongated neck portion. Some proliferating cells were also noted in the fundic glandular areas. In the stroma with chronic inflammatory cell infiltration, there were some Ki7 antigen-positive proliferating cells. In a germinal center of the lymph follicle there were many Ki67 antigen-positive proliferating cells. **c)** Intestinal metaplasia. The metaplastic glands demonstrated labeled proliferating cells in the base portion of the glands.

moderately inflamed mucosa showed moderate infiltration of chronic inflammatory cells in the enlarged stroma of the upper portion of the mucosa and lymphocytic aggregation forming lymph follicles in the deep portion of the mucosa (Table 1, Inflamed 2 and 3). As shown in Figure 2b, the severely inflamed mucosa showed severe lymphocytic infiltration in the enlarged stroma in the entire mucosa and marked lymph follicle formation in the deep portion of the mucosa (Inflamed 4 and 5 in Table 1). According to the criteria of the Sydney system, the Inflamed 1 to 5 lesions were evaluated as follows: HP: mild; neutrophils: normal; mononuclear cells: mild, moderate and marked; atrophy: normal (Inflamed 1 to 3) and moderate (Inflamed 4 and 5); intestinal metaplasia: normal.

In the metaplastic mucosa, HP was not seen. As shown in Figure 2c, one area showed residual fundic glands (Residual 1) and was covered by glandular epithelia with intestinal metaplasia (IM) (Metaplastic 1) as evaluated as followings; HP: normal; neutrophils: normal; mononuclear cells: normal; atrophy: normal; and intestinal metaplasia: mild to marked, according to the criteria of the Sydney system. The other area revealed complete replacement of the gastric fundic glands with intestinal metaplastic glands (Metaplastic 2).

Proliferating cells (Immunohistochemistry of Ki67 antigen)

The distribution of Ki67 antigen-positive cells in each glandular lesion of the gastric mucosa was evaluated.

As shown in Figure 3a, the hAR-polymer method of Ki67 antigen labeled some proliferating glandular epithelia in the neck portion of the glands (neck Ki67-positive cells) in the Normal 1 and the Inflamed 1 and 2. In the moderately inflamed mucosa (Inflamed 3) with HP infection, many neck

Ki67-positive cells were seen in a line in the elongated neck portion, reflecting the upward pipe-line system of the foveolar epithelia [21]. In the severely inflamed mucosa (Inflamed 4 and 5 and Residual 1), besides many neck Ki67-positive cells in the elongated neck portion, a few or some Ki67 antigen-positive cells were seen in the fundic glands (Fig. 3b) and suggested the stochastic flow system of the fundic glandular epithelia [21]. In the metaplastic mucosa (Metaplastic 1 and 2), Ki67 antigen-positive cells were found in the deep and base portion of the glands (Fig. 3c).

There were some Ki67 antigen-positive cells in the chronic inflammatory infiltrates (Fig. 3b).

Many Ki67 antigen-positive cells were seen in the germinal centers of the lymph follicles in the deep portion of the mucosa (mucosa-associated lymphoid tissue: MALT) and some were also noted in the marginal areas of the MALT (Fig. 3b).

Stem cells (Immunohistochemistry of CD34, CD117 and CD133)

In the immunostaining of the hAR-polymer method of CD34 and CD117, more CD34-positive cells besides blood vessel endothelia (Fig. 4a) were noted than CD117-positive cells (Fig. 4e). In the immunostaining of the hAR-supersensitive method of CD34, CD117 and CD133, more labeled cells were found in the order of CD117 (Fig. 4f), CD34 (Fig. 4b) and CD133 (Fig. 4g). Tissue stem cells of the bone marrow and hematopoietic stem cells could be labeled by means of the hAR-polymer or hAR-supersensitive method of these antibodies. On the other hand, peripheral blood stem cells in the PBTS were labeled by means of the hAR-polymer method of CD34 (Fig. 4c) and by means of the hAR-supersensitive method of CD34 (Fig. 4d) and CD133 (Fig. 4h). Therefore, the amount of CD133 in the paraffin sections of the ordinary fixed and processed tissue specimens was quite smaller than that of CD34. CD117 would appear in progenitor cells and hematopoietic blasts in the bone marrow and the CD117-positive peripheral blood stem cells were shown to be very rare.

The hAR-polymer method of CD117 labeled rare small and medium-sized cells in the mucosal stroma in a part of the almost normal gastric fundic mucosa (Table 3a, Normal 1). In parts of the upper portion of the moderately inflamed fundic mucosa (Table 3a, Inflamed 2), as shown in Figure 5b, the hAR-supersensitive method of CD117 labeled some positive cells in the neck portion (Neck CD117-positive cells) and somewhat aggregative distribution of a few CD117-positive cells among infiltrates. These CD117-positive cells were noted in the immunostaining of the hAR-polymer method (Table 3b, Inflamed 3). In the severely inflamed gastric fundic mucosa (Table 3a, Inflamed 4 and 5), the hAR-polymer method of CD117 labeled some cells in and around the neck portion (Fig. 5a) and a few fundic glandular cells (Fundic CD117-positive cells) (Fig. 5c). In IM,

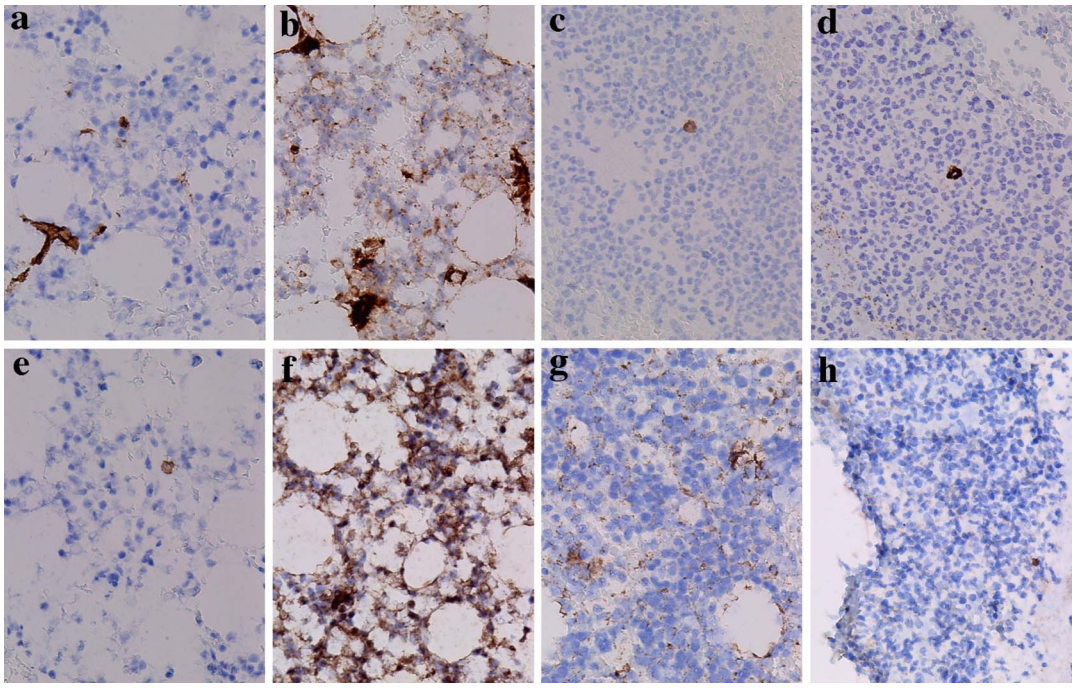


Fig. 4. Immunostaining of CD117, CD34 and CD133 for stem cells in the bone marrow and in the peripheral blood (×40 magnification). **a–d)** CD34. **e** and **f)** CD117. **g** and **h)** CD133. **a, b, e, f** and **g)** Aspiration clot specimen of the bone marrow. **c, d** and **h)** Peripheral blood tissue specimen (PBTS). **a, c** and **e)** hAR-polymer method. **b, d, f, g** and **h)** hAR-supersensitive method. In the bone marrow, beside CD34-positive blood vessel endothelia, immunostaining by hAR-polymer method of CD34 showed more positive cells than that of CD117, and immunostaining of hAR-supersensitive method showed more and stronger positive cells in the order of CD117 (**f**), CD34 (**b**) and CD133 (**g**). In the peripheral blood, a few CD34-positive cells (**c, d**) and CD133-positive cells (**h**) were noted.

Table 3a. *Immunohistochemical evaluation (Proliferation and stem cells)*

Lesion examined	Proliferation (Ki67 antigen)	Stem cells	
		CD117	CD34 (Besides capillary endothelia)
Normal 1	Some positive cells in the neck portion (Neck Ki67-positive cells) (Fig. 3a).	Rare small and medium-sized CD117-positive cells in the loose interglandular stroma.	A few flat epithelia in the neck portion, some faintly positive glandular cells and a few stromal positive cells (Supersensitive, Fig. 5e).
Normal 2	Not examined.	Not examined.	Not examined.
Inflamed 1	Many neck Ki67-positive cells.	No CD117-positive cells.	Some faintly positive glandular cells (Supersensitive).
Inflamed 2		Some CD117-positive cells in the neck portion (Neck CD117-positive cells). A few somewhat-aggregative CD117-positive cells among infiltrates (Aggregative CD117-positive cells) (Supersensitive, Fig, 5b).	
Inflamed 3	Many neck Ki67-positive cells in the elongated neck portion.	A few neck CD117-positive cells. A few aggregative CD117-positive cells.	Some faintly positive glandular cells (Supersensitive).
Inflamed 4	Many neck Ki67-positive cells in the elongated neck portion and a few Ki67-positive cells in the fundic glandular portion (fundic Ki67-positive cells).	Besides the neck CD117-positive cells, some CD117-positive cells in fundic glandular areas (Fundic CD117-positive cells). Some aggregative CD117-positive cells (Fig. 5a, c).	Many positive glandular cells and stromal positive cells (Supersensitive, Fig. 5f).
Inflamed 5	Many neck Ki67-positive cells in the elongated neck portion and some fundic Ki67-positive cells (Fig. 3b).	Besides neck CD117-positive cells, fundic CD117-positive cells and aggregative CD117-positive cells. Some CD117-positive cells around the neck portion (adhesive CD117-positive cells).	Same as above.
Residual 1	Same as above.	A few cells with faint or granular cytoplasmic stain of CD117 in the stroma.	Same as above (Fig. 5g).
Metaplastic 1	Some Ki67-positive cells in the deep to base portion of the glands (Fig. 3c).	Many faint-CD117-positive cells in the deep portion. Obviously CD117-positive cells in the base portion. A few cells with faint or granular cytoplasmic stain of CD117 in the stroma (Fig. 5d).	No positive glandular cells (Supersensitive).
Metaplastic 2	Same as above.	Same as above.	Same as above.

CD117-positive cells were seen at the base portion of the glands (Fig. 5d).

The hAR-polymer method of CD34 labeled blood vessel endothelia but did not label gastric glandular cells. By the hAR-supersensitive method of CD34, in the mostly normal mucosa (Table 3a, Normal 1), besides the blood vessel endothelia and spindle stromal cells, a few flat epithelia in the neck portion and some fundic glandular epithelia revealed faintly positive immunostaining (Fig. 5e). In the inflamed mucosa, many fundic glandular epithelia were labeled (Fig. 5f, g), whereas the foveolar epithelia and the glandular epithelia with IM were not labeled.

CD133 was not detected in the gastric fundic mucosa by the hAR-polymer method. However, in the hAR-supersensitive method, CD133 clearly labeled the cytoplasm of the gastric fundic glandular epithelia (Fig. 5h).

It was hard to evaluate how CD34 and CD133 label the gastric stem cells and gastric epithelial stem cells because many fundic glandular cells were labeled by the hAR-supersensitive method of CD34 and CD133. However, the hAR-polymer and hAR-supersensitive method of CD117 labeled the kinetics of the gastric stem cells and the gastric epithelial stem cells (Fig. 1). In Table 3a, CD117-positive stromal cells might be gastric stem cells that began to differentiate into the gastric epithelial or stromal stem cells in the Normal 1 (Table 3a) and in the Inflamed 2–5 (Table 3a) with the inflammatory environment, where several Ki67 antigen-positive cells were noted (Fig. 5b). Some adhesive CD117-positive cells around the neck portion in the Inflamed 5 may be the gastric stem cells differentiating into the gastric epithelial stem cells. Some neck CD117-positive cells in the elongated neck portion with many Ki67-positive cells in the Inflamed 2–5 seemed to be gastric epithelial stem cells that began to differentiate into the foveolar glandular cells. Some fundic CD117-positive cells in the Inflamed 4 and 5 and the Residual 1 (Table 3a) of the severely inflamed mucosa with fundic Ki67-positive cells may be the gastric epithelial stem cells that began to differentiate into the fundic glandular cells. The neck and fundic CD117-positive cells only in parts of the mucosa suggested that young gastric epithelial stem cells express CD117 in the very early phase of their differentiation into the gastric foveolar and

Table 3b. *Immunohistochemical evaluation (Programmed cell death)*

Glandular lesion examined	Muramidase	CD68	Programmed cell death	
			hAR and polymer method of cleaved casapase-3	eAR and supersensitive method of beclin-1
Normal 1	Strongly positive surface and neck cells and weakly positive fundic cells. Some positive stromal cells (Fig. 6a).	Some positive cells in the thin stroma in the upper portion and fewer positive cells in the deep portion of the mucosa.	Some foveolar positive epithelia below the surface (Foveolar positive cells) (Fig. 7b).	Weakly positive-fundic glandular cells just under the neck portion and nuclear stain in a few cells among them (Fig. 8a).
Normal 2	Not examined.	A few positive cells in the loose stroma in the deep portion.	Relatively many foveolar positive cells.	Weakly positive-fundic glandular cells just under the neck portion.
Inflamed 1	Strongly positive entire glandular cells. Some positive stromal cells.	Many aggregative positive cells among the infiltrates.	A few foveolar positive cells.	Same as above in parts.
Inflamed 2	Same as above.	Same as above.	A few foveolar positive cells.	Same as above in parts (Fig. 8d).
Inflamed 3	Same as above.	Same as above.	A few foveolar positive cells and a few sloughed positive cells in the glandular lumen.	Same as above.
Inflamed 4	Same as above.	Not examined.	A few foveolar positive cells and a few positive cells in the neck portion (Neck positive cells).	Very weakly positive fundic glandular cells and some strongly positive fundic cells (Fig. 8c).
Inflamed 5	Same as above (Fig. 6b).	Many aggregative positive cells among the infiltrates.	Many foveolar positive cells and some neck positive cells (Fig. 7d, e).	Same as above, especially among dense infiltrates.
Residual 1	Same as above but many positive stromal cells.	Some positive cells in the stroma.	Positive cells in the stroma.	Some positive fundic glandular cells and nuclear stain in a few cells.
Metaplastic 1	Weakly positive surface cells and strongly positive basal portion cells.	Cell apex and supranuclear stain in the surface cells and granular supranuclear stain in the base portion (Fig. 6c).	Many positive surface metaplastic epithelia.	Negative.
Metaplastic 2	Same as above.		Same as above.	Negative.

glandular cells.

Lysosome and macrophage (Immunohistochemistry of muramidase and CD68)

The activation of lysosomes in the glandular epithelia and the existence of macrophages in the glandular lumen were investigated here. Gastric fundic glandular cells that had fallen into programmed cell death with membrane-boundary apoptotic bodies were reported to be processed by heterophagy of the neighboring glandular epithelial cells and macrophages, or to be released into the glandular lumen [44].

As shown in Table 3b, in the mostly normal gastric fundic mucosa, the glandular epithelia that were located from the surface to the neck portion of the gastric glands were strongly positive for muramidase (Fig. 6a), whereas those in the entire mucosa were positive in the inflamed mucosa (Fig. 6b).

As shown in Table 3b, in the gastric fundic mucosa, no CD68-positive macrophages were found in the gastric glands, but they were noted in the stroma among the gastric glands. The glandular epithelia with IM expressed CD68 in the cytoplasm or in the cell apex (Fig. 6c).

The lysosomes were shown to be activated in the glandular cells of the inflamed mucosa without any macrophages in the glandular lumen.

Apoptosis (Immunohistochemistry of ssDNA and cleaved caspase-3)

Single-stranded DNA, a marker for the late process of apoptosis [12], appears in the DNA fragmentation factor (DFF) catalysis of double-stranded DNA. The appearance of cleaved caspase-3 is the first molecular event in the irreversible reaction of apoptosis [10, 15, 45]. The eAR-polymer method of ssDNA labeled almost all the nuclei in the gastric mucosa probably as signs of DNA damage and showed cytoplasmic staining in the areas of parietal cells probably as the effect of gastric acid juice (Fig. 7a). The hAR-polymer method of cleaved caspase-3 [13] labeled some or relatively many foveolar epithelia below the surface in the mostly normal mucosa (Table 3b, Normal 1 and 2, Fig. 7b), suggesting inhibiting apoptosis in the surface epithelia under HP infection [32]. Its hAR-supersensitive method labeled only weakly the foveolar epithelia, but strongly

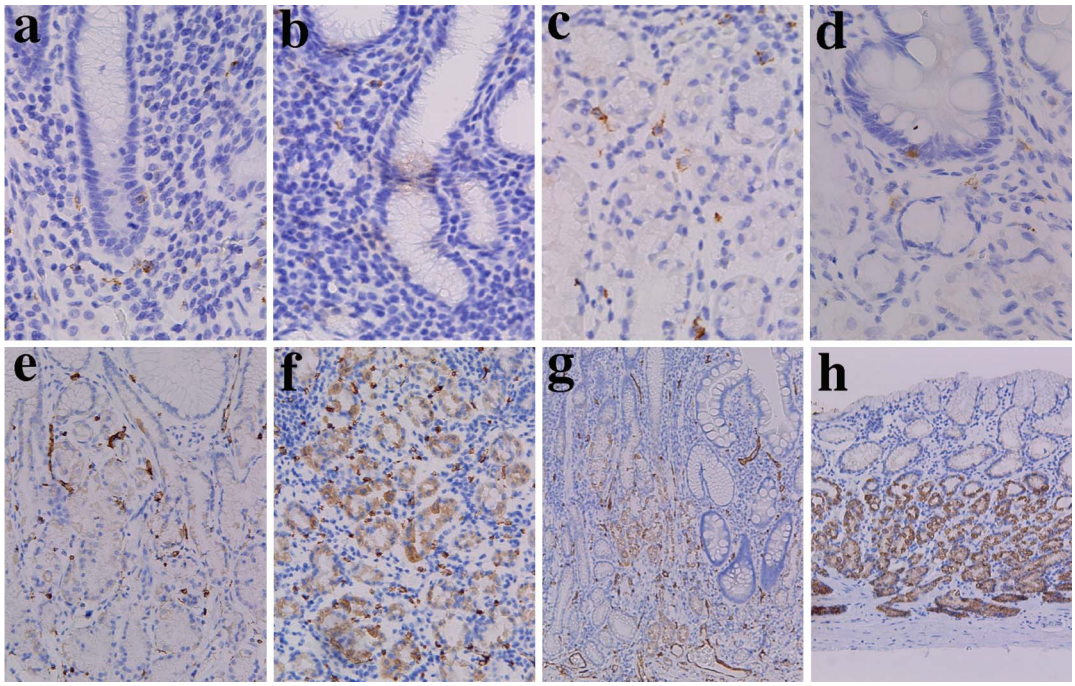


Fig. 5. Immunostaining of CD117, CD34 and CD133 for stem cells in the gastric mucosa. **a–d)** CD117. **a)** Inflamed mucosa (hAR-polymer method, ×40 magnification). CD117-positive cells were noted in and around the neck portion and in the stroma with dense chronic infiltrates. **b)** Inflamed mucosa (hAR-supersensitive method, ×40 magnification). A trace expression of CD117 in gastric glandular cells at the neck portion. **c)** Fundic glands in inflamed mucosa (hAR-polymer method, ×40 magnification). Some CD117-positive fundic glandular cells under the neck portion. **d)** Intestinal metaplasia (hAR-polymer method, ×40 magnification). CD117-positive cells in the base portion of a metaplastic gland. **e–g)** CD34. **e)** Inflamed mucosa (hAR-supersensitive method, ×20 magnification). Besides blood vessel endothelia, CD34-positive fat cells and glandular cells were seen but most fundic glandular cells were labeled very weakly. **f)** Fundic glands in the inflamed mucosa (hAR-supersensitive method, ×20 magnification). Fundic glandular cells were labeled by CD34. **g)** Intestinal metaplasia (hAR-supersensitive method, ×10 magnification). Residual fundic glandular cells in metaplastic mucosa were also labeled by CD34. **h)** CD133. Mostly normal mucosa (hAR-supersensitive method, ×10 magnification, long axis: 680 μm). Fundic glands were labeled densely by CD133.

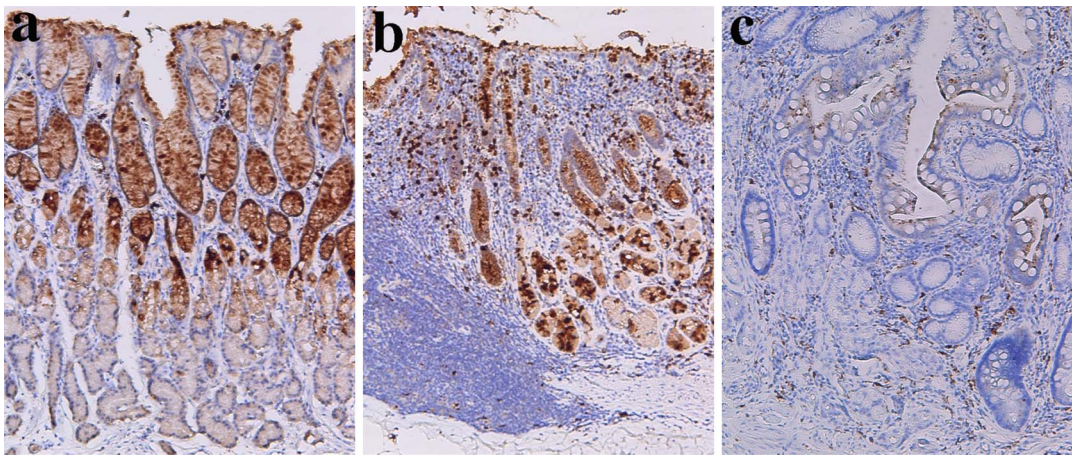


Fig. 6. Immunostaining of muramidase and of CD68 (hAR-polymer method, ×10 magnification). **a)** Muramidase in the mostly normal mucosa. Muramidase was noted in glandular cells from the surface to the neck portion. **b)** Muramidase in inflamed mucosa. Muramidase was also noted in deep fundic glandular cells. **c)** CD68 in an area of intestinal metaplasia. There were CD68-positive cells in the stroma and CD68-positive metaplastic glandular cells.

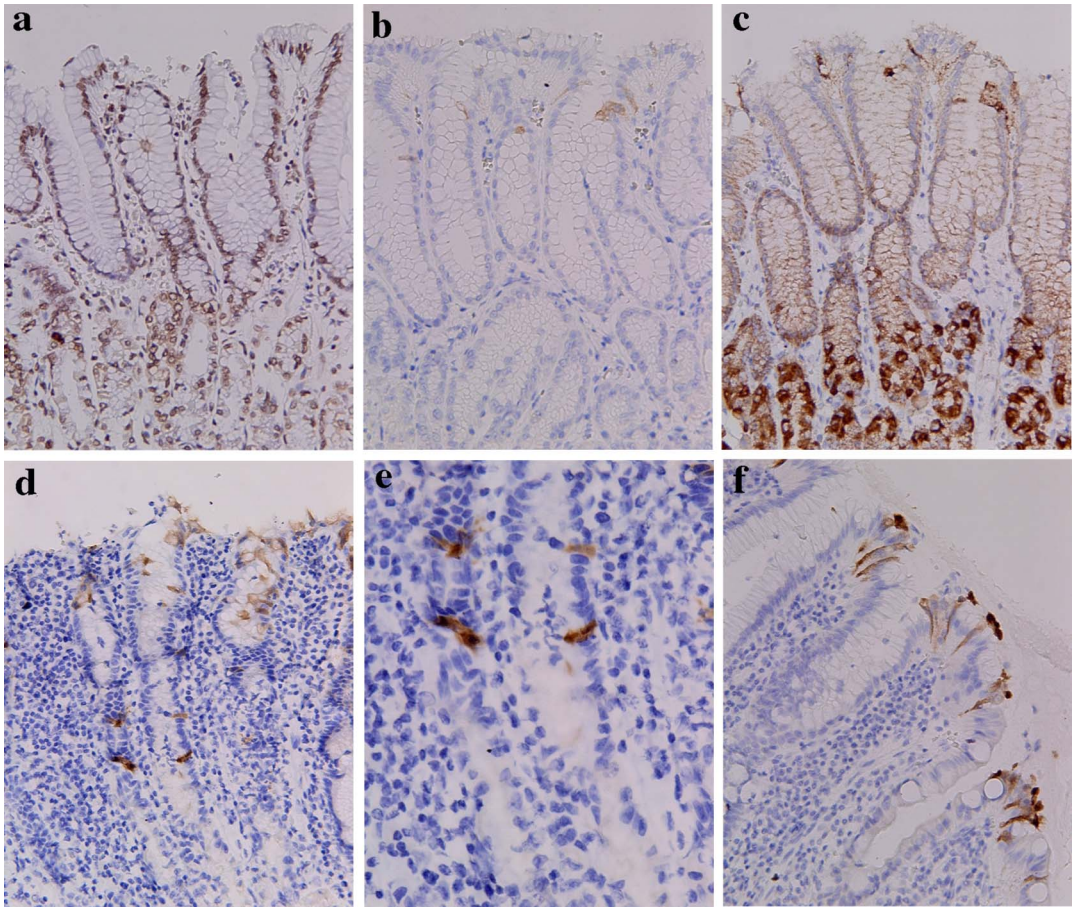


Fig. 7. Immunostaining of single-stranded DNA (ssDNA) and cleaved caspase-3 for apoptosis in the gastric mucosa. **a)** eAR-polymer method of ssDNA in the mostly normal gastric mucosa (×20 magnification). Anti-ssDNA antibody labeled most of the nuclei of the glandular cells. **b)** hAR-polymer method of cleaved caspase-3 in the mostly normal gastric mucosa (×20 magnification). Foveolar cells under the surface were positive for cleaved caspase-3. **c)** hAR-supersensitive method of cleaved caspase-3 in the mostly normal gastric mucosa (×20 magnification). Labeled cells other than those labeled by hAR-polymer method (**b**) were thought to degrade the prepared caspase-3. **d)** hAR-polymer method of cleaved caspase-3 in inflamed mucosa (×20 magnification). Relatively many cleaved caspase-3-positive foveolar cells under the eroded surface. Some cleaved caspase-3-positive cells were also seen in the neck portion. **e)** hAR-polymer method of cleaved caspase-3 in the neck portion of the inflamed mucosa (×20 magnification). Some cleaved caspase-3-positive cells in the neck portion where many proliferation cells were located. **f)** hAR-polymer method of cleaved caspase-3 in intestinal metaplasia (×20 magnification). Surface cells of the metaplastic glands were positive for cleaved caspase-3.

labeled some fundic glandular epithelia, probably parietal cells (Fig. 7c), besides the cells labeled in the hAR-polymer method. The hAR-polymer method using cleaved caspase-3 is thus indicated to be the best method for labeling apoptosis [13].

In the inflamed gastric mucosa (Table 3b, Inflamed 1–5), a few or some foveolar epithelial cells under the surface were labeled by the hAR-polymer method of cleaved caspase-3 (Fig. 7d), thus indicating that the labeled apoptosis represented apoptosis occurring after their lifetime under inhibiting apoptosis by HP infection [32]. In the Inflamed 4 and 5 (Table 3b) there were a few cleaved caspase-3-positive cells in the elongated neck portion (Fig. 7d, e) with many Ki67-positive proliferating cells (Fig. 3b), suggesting that damaged proliferating cells differentiating into the foveolar cells that had fallen into apoptosis. The surface epithelia in the gastric mucosa with IM were positive for cleaved caspase-3 (Fig. 7f), thus indicating that the labeled apoptosis was synchronous after their lifespan. However, the apoptosis of the gastric fundic glandular cells was not detected by means of the hAR-polymer method of cleaved caspase-3.

Autophagy (Immunohistochemistry of beclin-1)

The AR-polymer method of beclin-1 was found not to label any cells. However, the hAR-supersensitive method of beclin-1 showed much stronger staining (Fig. 8b) than the eAR-supersensitive method (Fig. 8a), indicating the autophagy in the gastric fundic glandular epithelia. However, the eAR-supersensitive method of beclin-1 could differentiate the nuclear staining of beclin-1 [29] from the cytoplasmic staining (the inserted figure in Fig. 8a), indi-

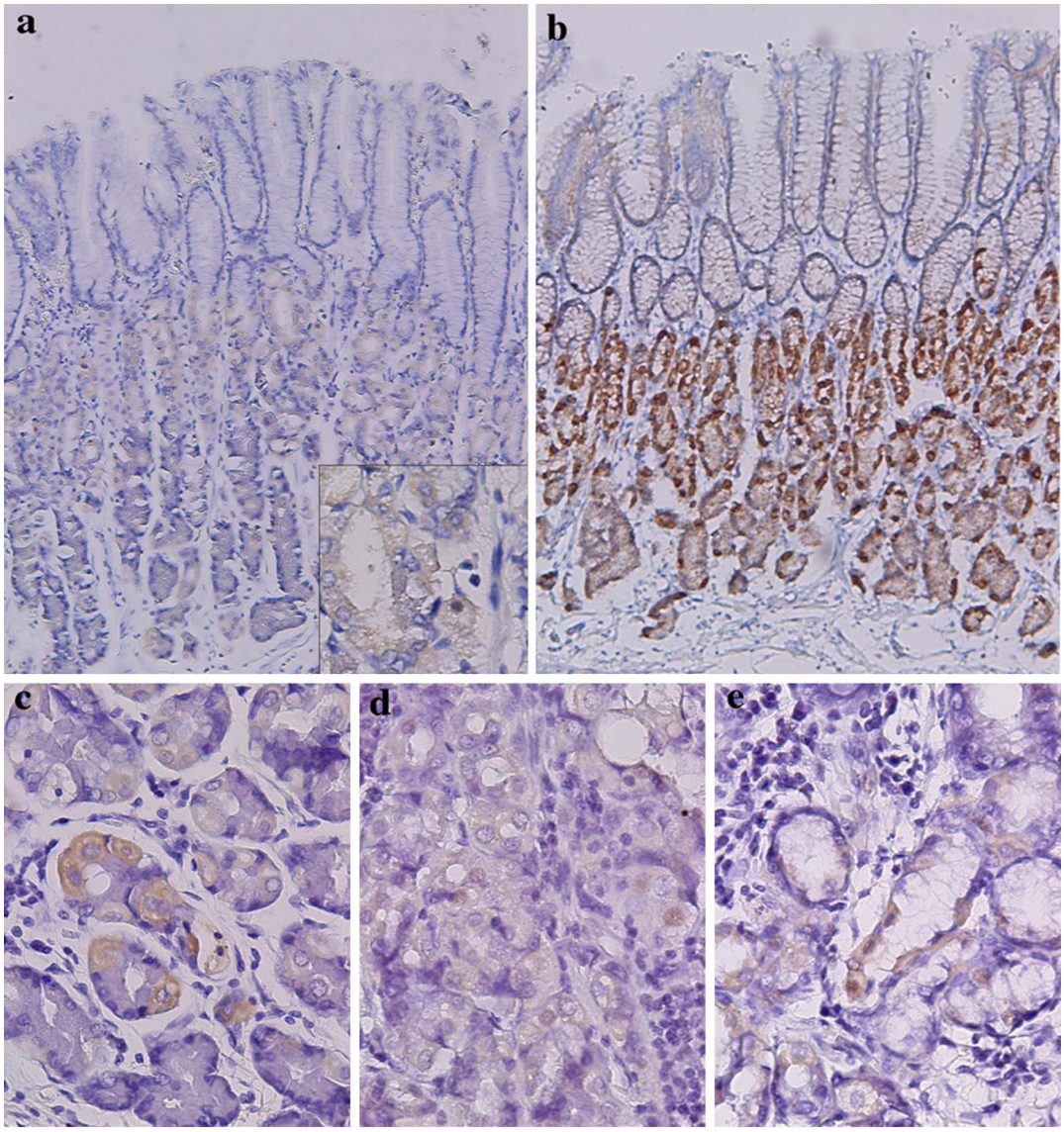


Fig. 8. Supersensitive immunostaining of beclin-1 in the mostly normal and inflamed gastric mucosa. **a)** eAR-supersensitive method (×10 magnification). A beclin-1-positive nucleus was noted in the inserted figure (×20 objective and ×6.4 digital enlargement, length of axis was 67 μm). **b)** hAR-supersensitive method (×10 magnification). Foveolar cells were weakly labeled and fundic glandular cells were densely stained. **c)** eAR-supersensitive method (×40 magnification). A fundic gland included some beclin-1-positive cells and some of them were degenerated. **d)** eAR-supersensitive method (×40 magnification). In fundic glands under the neck portion some nuclei were positive for beclin-1. **e)** eAR-supersensitive method (×40 magnification). In fundic glands under the neck portion, an atrophic gland showed glandular cells which revealed cytoplasmic and nuclear staining of beclin-1.

cating that the eAR-supersensitive method of beclin-1 can monitor the mutated beclin-1 [29].

In the eAR-supersensitive method of beclin-1, some fundic glandular cells showed strongly positive staining in the cytoplasm in the severely inflamed mucosa (Table 3b, Inflamed 4 and 5). Some of the fundic glandular cells were degenerative (Fig. 8c, e), thus indicating that a few cells had entered a state of autophagic cell death in the inflamed mucosa. In parts of the gastric fundic glandular areas under the neck portion, the nuclear staining of beclin-1 was noted (Fig. 8a, d, e) without obvious relation to the inflammation.

IV. Discussion

This study analyzed the homeostatic mass control in the fundic glands in the stomach with cancer, while maintaining the two-layered structure of the foveolar part and the fundic glandular part.

There were more Ki67 antigen-positive proliferating cells [51] concentrated in the elongated neck portion and scattered in the fundic glandular portion in the inflamed mucosa under HP infestation than in the mostly normal mucosa. This reflected the upward pipe-line system of the foveolar

epithelia and the downward stochastic flow system of the fundic glandular epithelia [21] and at the same time suggested that HP infestation propels gastric epithelial proliferation.

This study showed that the peripheral blood stem cells in the homeostatic growth of adult tissue cells (Fig. 1a) were labeled by CD34 [11] and CD133 [1] but not by CD117 [24, 31, 42]. CD34 and CD133 were thought to be markers of pluripotent stem cells. However, CD34 labels blood vessel endothelia and CD133 labels the other cells [1]. On the other hand, CD117 labeled strongly much more hematopoietic cells in the bone marrow and a small number of stromal cells in the gastric mucosa, some proliferating cells in the neck portion and in the fundic glandular portion of the inflamed gastric mucosa under HP infection. It was reported in children with congenital heart disease that there were CD117 (c-kit)-positive progenitor cells differentiating into cardiac muscle cells [43]. CD117 and CD45 double positive cells and CD117 and GATA-4 (early marker of cardiomyocytes) were recognized. Since extramedullary hematopoiesis was usually not observed even in the hearts of such children, CD117-positive stem cells differentiating into cardiomyocytes might transiently express CD45 in the early phase of their differentiation. In this study, there were CD117-positive progenitor cells from the gastric stem cells to the gastric epithelial stem cells in the gastric mucosal stroma, as well as Ki67-positive proliferating and CD117-positive transient cells from the gastric epithelial stem cells to the terminal differentiated gastric glandular cells in the neck portion in the adult homeostatic growth of the gastric mucosa. Therefore, HP infection evokes gastric inflammation on the one hand and indirectly propels the supply of gastric epithelial stem cells and their differentiation to the mature cells on the other. In addition, gastric epithelial stem cells for IM [48] with the expression of CD68, shown in this study, might be the product of an altered extracellular microenvironment in the neck portion of the gastric mucosa under HP infection.

As for apoptosis, the molecular mechanism from death receptors, mitochondria route and inhibitors have been elucidated. Cleaved caspase-3 is an effector caspase that starts the irreversible downhill pathway of apoptosis [10, 15, 45]. Thus, the IHC of cleaved caspase-3 was developed [13, 52]. The nonspecific staining of ssDNA in the gastric mucosa (Fig. 7a) suggested that the hAR-polymer method of cleaved caspase-3 [13] is superior in detecting apoptosis to the eAR-polymer method of ssDNA [36]. Difficulty in detecting apoptosis by histochemical labeling the apoptosis end-product fragmented DNA such as TUNEL (TdT-mediated dUTP-biotin Nick End Labeling) method has been shown in some pathological conditions. In rheumatoid arthritis, inflammatory superoxides-induced DNA fragmentation and DNA damage in the synoviocytes are labeled by such methods. However, their apoptosis is really suppressed by the activation of FLICE/caspase-8 inhibitory protein [40, 47]. HP itself and the gastric glandular epithelia under HP infection probably produce superoxide radicals such as nitric oxide [16, 35]. Therefore, the nonspecific staining of ssDNA in Figure 7a may be due to the DNA damage in the gastric epithelial nuclei under HP infection as well as post-resection effects of the residual gastric juice and the long-term storage effects of the specimens. This study compared the hAR-polymer and hAR-supersensitive methods of cleaved caspase-3. As shown in Figure 7b, the hAR-polymer method of cleaved caspase-3 labeled clearly the apoptotic cells. The hAR-supersensitive method of cleaved caspase-3 labeled the foveolar epithelia weakly and some fundic glandular cells, probably parietal cells (Fig. 7c) strongly besides the apoptotic cells labeled by the hAR-polymer method. Apoptotic signals, such as activated caspase-8, cleave prepared caspase-3 into cleaved caspase-3 and the un-cleaved caspase-3 is degraded. A very small amount of degraded caspase-3 may be detected by the hAR-supersensitive method and the residual gastric juice in parietal cells enhanced the labeling, because partially degraded caspase-3 could be labeled by anti-cleaved caspase-3 polyclonal antibody [25].

The apoptosis in the foveolar epithelia just under the surface of the mucosa was regarded as that after the lifetime in the upward pipe-line growth system of the foveolar epithelia when HP suppresses apoptosis in the surface epithelia [32]. HP itself was not recognized in the H&E-stained almost normal gastric mucosa but the IHC detection of such foveolar epithelial apoptosis suggested HP infection. On the other hand, the sporadic apoptosis in the neck portion in the severely inflamed mucosa under HP infection suggested the apoptosis of proliferating cells damaged by superoxide radicals or by a cross signaling of proliferation and apoptosis [8] induced by some unknown mechanism. In the fundic glands there were no apoptotic cells labeled by the hAR-polymer method of cleaved caspase-3, thus suggesting the existence of some other mechanisms of programmed cell death in the fundic glandular epithelia.

The other form of programmed cell death is autophagy, which comprises macroautophagy for organelles, microautophagy for altered proteins and the others, and chaperone-related autophagy. The terminal phase of macroautophagy is thought to be autophagic cell death [37]. Autophagy other than autophagic cell death is one of the cellular functions to survive under starvation and the other strong stimuli. In the human stomach, autophagolysosomes were reported in an electronmicroscopic study [38]. Up to now no sufficient morphological method for labeling autophagy has been available [34]. However, autophagic vesicles incorporate lysosomes and form autophagolysosomes, which could be visualized by means of the IHC of lysosomes, such as the hAR-polymer method of muramidase in this study. The lysosomes detected in the inflamed mucosal fundic glandular epithelia were thus thought to be autophagolysosomes rather than phagosomes (secondary lysosomes) demonstrating immunity against the HP infection. The expression of beclin-1 corresponding with Apg6 in yeast increased the number of autophagic vesicle in MCF7 breast cancer cell lines [28]. Using the anti-beclin-1 antibody supplied commercially, we first succeeded in detecting beclin-1 on the paraffin sections of routinely processed human non-neoplastic tissue specimens using the hAR- or eAR-supersensitive method. The

hAR-supersensitive method seemed to detect autophagolysosomes. However, depending probably on the amount of beclin-1, the eAR-supersensitive method of beclin-1 could label an abnormal nuclear expression of beclin-1 [29] and autophagic cell death.

The nuclear expression of beclin-1 was recognized in the fundic glandular part under the neck portion of the almost normal and severely inflamed gastric mucosa (Table 3b). As Liang *et al.* reported [28, 29], the beclin-1 molecules may be mutated and lose their functions for autophagy and tumor suppression. Therefore, the nuclear expression of beclin-1 is one of the representative findings suggesting genetic alterations in the gastric fundic glandular cells under HP infection in spite of no obvious relation between the nuclear expression of beclin-1 and the inflammation.

In the severely inflamed gastric fundic mucosa, some cells in a few fundic glands were strongly positive for beclin-1 in the immunostaining by the eAR-supersensitive method and were atrophic and degenerative, suggesting that the fundic glandular cells went into autophagic cell death after their lifetime rather than that under HP infestation [3]. The programmed cell death in the gastric fundic glandular cells was thus autophagic cell death rather than the apoptosis with membrane-boundary apoptotic bodies [44]. When there were no CD68-positive macrophages in the gastric glands, the gastric glandular cells that went into autophagic cell death would be excreted through the glandular lumen to the gastric lumen.

The AR-supersensitive method can save the primary antibody on the one hand and can detect an extremely small amount of antigens on the other. In this study, we applied the AR-supersensitive method [17, 20] in detecting CD117, CD34, CD133, cleaved caspase-3 and beclin-1. In the case of CD117, the hAR-supersensitive method could detect a very less expression of CD117 in the transient cells derived from the young gastric epithelial stem cells in the neck portion than the hAR-polymer method. But in the cases of CD34 and CD133, it was hard to evaluate their extremely low expression in the tissue as the stem cells' phenotype in the staining by the AR-supersensitive method, because their properties other than stem cells' phenotypes were strongly stained. In the case of cleaved caspase-3, its hAR-supersensitive method labeled intermediate products in the degradation of caspase-3, as mentioned above, suggesting that the AR-supersensitive method can see a degradation phase of a target molecule. In the case of beclin-1, its eAR-supersensitive method could differentiate its expression in nucleus and cytoplasm whereas its hAR-supersensitive method showed too strongly positive staining to differentiate its expression in cytoplasm and nucleus, as mentioned above. The AR in the degree and specificity for detecting a target molecule is also important in the AR-supersensitive method according to the purpose of its application.

As for the pathogenicity of HP infection, the HP-suppressing apoptosis in the surface epithelia [32] was recognized as the apoptosis in the foveolar epithelia just under the surface from the viewpoint of homeostatic mass control. Furthermore, this study found some apoptotic proliferating cells in the neck portion and nuclear accumulation of beclin-1 losing its autophagy and tumor suppression functions [29] in a few fundic glandular cells just under the neck portion. These findings suggested the occurrence of genetic alterations in the neck cells and the fundic glandular cells just under the neck portion, where the components of HP itself, such as LPSs, and acute and chronic inflammation with anti-HP immunity comprise the microenvironment (Fig. 1b). There, gastric epithelia differentiate from the gastric epithelial stem cells. The CD117 expression was noted in two steps of the differentiation of the gastric epithelia, such as the transient cells from the young gastric epithelial stem cells and the progenitor cells from the gastric stem cells. Such differentiation steps may be the targets of the HP-related carcinogenesis in the microenvironment. The above-mentioned apoptosis in the proliferating neck cells and the nuclear expression of beclin-1 reinforces this view. Considering the homeostatic mass control of the gastric epithelia with the target differentiation steps of the HP-related carcinogenesis, the gastric carcinogenesis of the bone marrow-derived stem cells needs rare stem cells that have acquired at least the genetic and molecular alterations of the initiating step of the gastric carcinogenesis prior to entering the stomach. Therefore, we believe that this study suggested the *in-situ* occurrence of gastric cancer under HP infection.

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VI. References

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