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Apoptotic cell death and its relationship to gastric carcinogenesis

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Abstract

AIM: To investigate the apoptotic process of cells within the intestinal metaplasia areas co-localizing with chronic gastritis and gastric carcinomas and to analyze the involvement of proteins regulating apoptosis in the process of intestinal metaplasia related gastric carcinogenesis.

METHODS: Forty-two gastric carcinoma and seventeen chronic gastritis cases were included in this study. All cases were examined for the existence of intestinal metaplasia. Ten cases randomly selected from each group were processed for TUNEL assay. TUNEL positive cells within the intestinal metaplasia areas, co-localizing either to gastric carcinoma or chronic gastritis, were counted and converted to apoptotic indices. In addition, p53, bcl-2 and bax expression patterns within these tissues were analyzed on the basis of immunohistochemistry.

RESULTS: Twenty-eight of the cases were intestinal and 14 of the cases were diffuse type adenocarcinomas. 64% (27/42) of the gastric carcinoma cases had intestinal metaplasia. Intestinal metaplasia co-localized more with intestinal type carcinomas compared with diffuse type carcinomas [75% (21/28) *vs* 42% (6/14), respectively; $P \leqslant 0.05$]. The mean apoptotic index in tumor cells was 0.70 ± 0.08. The mean apoptotic index in intestinal metaplasias co-localizing to tumors was significantly higher than that of intestinal metaplasias co-localizing to chronic gastritis (0.70 ± 0.03 *vs* 0.09 ± 0.01, respectively; $P \leqslant 0.05$). p53 positivity was not observed in areas of intestinal metaplasia adjacent to tumors or chronic gastritis. Intestinal metaplasia areas adjacent to tumors showed lower cytoplasmic bcl-2 positivity compared to intestinal metaplasia areas adjacent to chronic gastritis [55.5% (15/27) *vs* 70.5% (12/17), respectively]. On the other hand, intestinal metaplasia areas adjacent to tumors showed significantly higher cytoplasmic bax positivity compared to intestinal metaplasia areas adjacent to chronic gastritis [44.4% (12/27) *vs* 11.7% (2/17), respectively; $P \leqslant 0.05$].

CONCLUSION: Existence of apoptotic cells on the basis of TUNEL positivity is shown in intestinal metaplasias co-localizing to both diffuse and intestinal type gastric cancers in this study. Our results also suggested bax expression dependent induction of apoptosis especially in intestinal metaplasia areas adjacent to tumors. These findings strongly support the involvement of apoptotic mechanisms in the process of gastric carcinogenesis especially in the transition from intestinal metaplasia to gastric cancer. It may be suggested that induction of apoptosis in intestinal metaplasia areas adjacent to tumors may involve different mechanisms than induction by chronic inflammation.

Key words: p53; Bax; Bcl-2; TUNEL staining; Intestinal metaplasia; Apoptosis; Gastric cancer

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INTRODUCTION

Studies by Correa have suggested that development of gastric cancer is a multistep process involving a series of events such as atrophic gastritis, intestinal metaplasia, and dysplasia. According to this model, these lesions are considered to be precancerous[1].

The cellular turnover of the gastric tissue and the balance within this unstable tissue is maintained by the processes known as proliferation and apoptotic cell death[2,3]. However, the apoptotic cell death plays an important role in the regulation of pathological conditions as well, such as development and progression of malignant tumors[3,4]. Among the methods that are used to identify

cells undergoing the apoptotic process, the TUNEL technique is one of the most successfully utilized[4].

Gastric epithelium is exposed to damaging agents in nature[2]. These agents may result in DNA damage in these epithelial cells. In response to DNA damage and other stress, p53 is activated by several post transcriptional modifications, which in turn activates its down-stream targets to induce cell cycle arrest or apoptosis. An arrested cell cycle allows the repair of the DNA damage. However, failure of the repair of the DNA damage results in activation of apoptosis by p53. Thus, p53 has a major tumor suppressor role in this process and mutations that abolish p53 function may fail to control this mechanism[5] and induce excessive cell proliferation and/or decreased cell apoptosis, which seem to be the biological basis of gastric carcinogenesis[3].

Bcl-2 family proteins are important apoptotic regulators and can act either pro- or anti-apoptotically. The bcl-2 gene product codes for anti-apoptotic protein, whereas the bax and bad gene products act pro-apoptotically. p53 has also been suggested for deregulation of cell death through Bax-Bcl-2 imbalances[5].

Intestinal metaplasia has been extensively studied as a possible premalignant condition in the human stomach[1,6-8]. Moreover, many questions remain regarding pathogenesis of intestinal metaplasia development as well as its relationship to gastric cancer. In this study, we have investigated immunohistochemical expression of apoptosis related proteins p53, bax and bcl-2 and TUNEL staining in the areas of intestinal metaplasia adjacent to gastric cancer and chronic gastritis in order to determine the apoptotic bodies to understand their role in gastric carcinogenesis.

MATERIALS AND METHODS

Paraffin-embedded biopsies or surgical specimens from 42 cases with gastric carcinomas and 17 cases with intestinal metaplasia and chronic atrophic gastritis were assessed in the Pamukkale University School of Medicine Department of Pathology (Figure 1A and B). Clinical characteristics of these patients were described in Table 1. Histological diagnoses and classification of tumors were based on Lauren's criteria[9] and intestinal metaplasia, chronic atrophic gastritis were diagnosed according to the updated Sydney system[10]. Intestinal metaplasias were classified according to Filipe *et al*[11].

Immunohistochemistry

Sections from each representative specimen were cut at 3-5 μm, mounted on glass and dried overnight at 37℃. Briefly, all sections were then deparaffinized in xylene, dehydrated through a graded series of alcohol and washed in distillated water. Distillated water was used for all subsequent washes and dilution of the antibodies. Tissue sections were heated twice in a microwave oven for 10 min each at 700 W in citrate buffer pH 6 and then processed with the standard streptavidin-biotin-immunoperoxidase method with automatically Ventana-Nexes immunostainer (Ventana Medical Systems, Tucson, Arizona). Monoclonal mouse anti-human p53 protein antibody (Clone DO-7, Neomarkers, Inc. 47790 Westinghouse Dr., Fremont CA 94539, USA) at a 1/50 dilution, monoclonal mouse anti-human antibody anti-Bcl-2 (Clone 7D9, Neomarkers, Inc. 47790 Westinghouse Dr., Fremont CA 94539, USA) at a 1/50 dilution, rabbit polyclonal immune serum raised against bax (Clone 2D2, Neomarkers, Inc. 47790 Westinghouse Dr., Fremont CA 94539, USA) at a 1/20 dilution were used. Diaminobenzidine was used as the final chromogen, and Mayer's haematoxylin as the nuclear counter stain.

Table 1 Clinical characteristics of cases

	Intestinal metaplasia (*n*)	Sex (*n*)		Age of cases (mean)	Number of cases (*n*)
		Female	Male		
Chronic gastritis	17	7	10	53.6 ± 16.0	17
Intestinal type adenocarcinoma	21	10	18	63.2 ± 10.3	28
Diffuse type adenocarcinoma	6	7	7	57.4 ± 16.6	14
Adenocarcinoma	27	23	19	53.5 ± 12.8	42

Sections were evaluated for the staining of p53, bcl-2 and bax proteins semi quantitatively. The protein expression in each specimen is scored for the percentage of positive neoplastic cells and intestinal metaplasia cells: score 0 = undetectable staining or < 20% of positive cells; score 1 = ⩾ 20 positive cells. Nuclear staining for p53 and cytoplasmic staining for bax and bcl-2 were scored. Lymphocytes and small vessels were used as positive controls for bcl-2 and bax immunoreactivity, respectively.

Detection of apoptosis

Apoptotic activity was analyzed on the basis of terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay. Sections from the same blocks of selected cases were cut at 10 μm, and mounted on glass. These sections were processed with the *In Situ* Cell Death Detection Kit, AP (Roche, Penzberg, Germany) according to the manufacturer's protocol for paraffin-embedded tissues. BM Purple AP Substrate, precipitating (Roche, Penzberg, Germany) chromogenic substrate was used for alkaline phosphatase enzyme immunoassay at the last step. We sought positive TUNEL staining against background of nuclear staining of gastric cancer and intestinal metaplasia cells.

Statistical analysis

SPSS software (version 10.00) was used for statistical analysis. Pearson's Chi-square test or Fisher's exact test were applied to analyze the data when appropriate. TUNEL staining results were statistically evaluated using the test for difference between two population proportions. $P < 0.05$ was taken as statistically significant.

RESULTS

Gastric carcinoma cases were assessed according to Lauren's classification and 28 intestinal, 14 diffuse type adenocarcinomas were included in this study. Intestinal

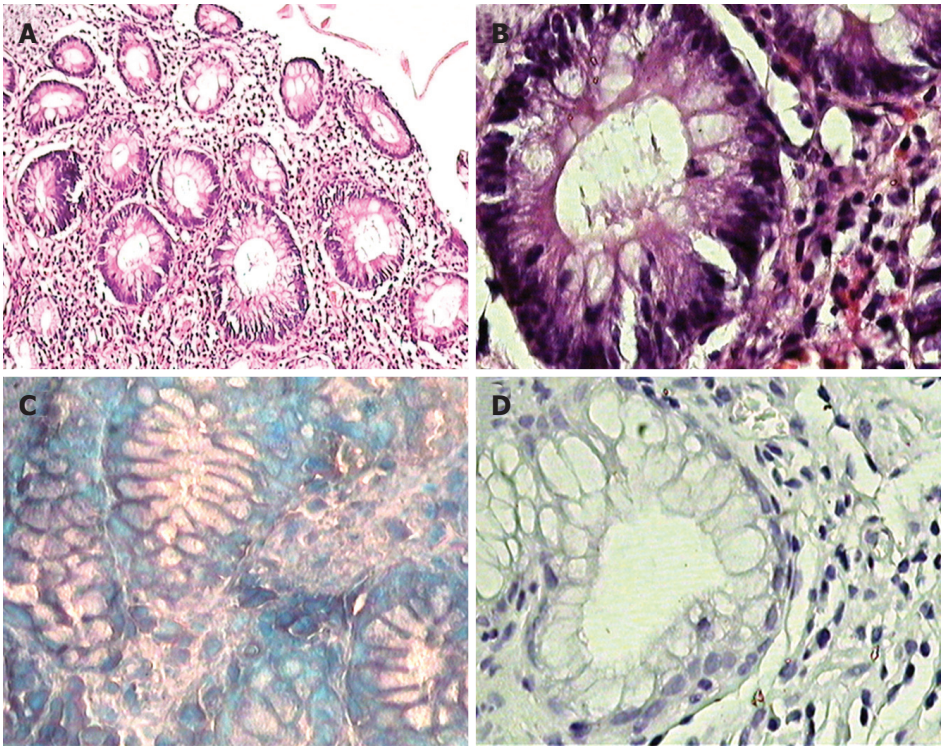

Figure 1 **A**: Chronic gastritis with intestinal metaplasia (HE, x 10); **B**: Areas of intestinal metaplasia in chronic gastritis (HE, x 40); **C**: A few intestinal metaplastic cells adjacent to the chronic gastritis showed positivity for TUNEL staining; **D**: Intestinal metaplasia areas adjacent to chronic gastritis without cytoplasmic bax positivity.

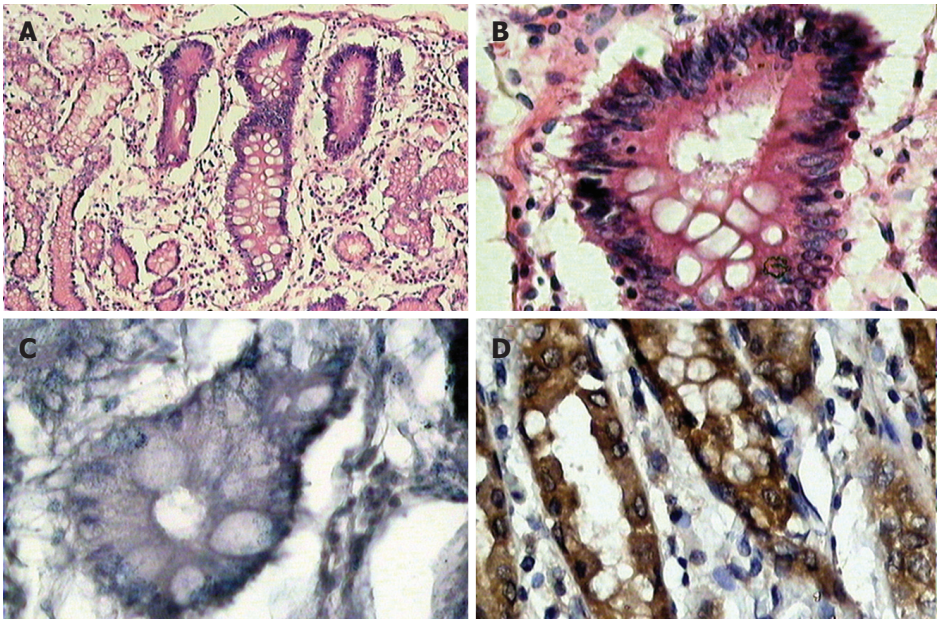

Figure 2 **A**: Adenocarcinoma with intestinal metaplasia (HE, x 10); **B**: Areas of intestinal metaplasia in adenocarcinoma (HE, x 40); **C**: Abundant intestinal metaplastic cells adjacent to the adenocarcinoma showed positivity for TUNEL staining; **D**: Intestinal metaplasia areas adjacent to adenocarcinoma with strong cytoplasmic bax positivity.

metaplasia was observed in 75% (21/28) of intestinal type carcinoma cases, 42% (6/14) of diffuse type carcinoma cases and 64% (27/42) of the overall gastric carcinoma cases (Figure 2A and B). Intestinal metaplasia areas were co-localized more with intestinal type carcinomas compared with diffuse type carcinomas and the difference was statistically significant ($P \leqslant 0.05$). Intestinal metaplasias co-localizing to tumors were classified according to Filipe and are shown to be 93% (25/27) Type Ⅰ, and 8% (2/27) Type Ⅱ; on the other hand, intestinal metaplasias co-localizing to chronic gastritis were all Type Ⅰ.

Randomly selected cases of tumor with adjacent intestinal metaplasia and ten cases of chronic gastritis with adjacent intestinal metaplasia were stained by the TUNEL method for the determination of apoptotic nuclei. Variable numbers of tumor cells were shown to have apoptotic nuclei in all of the tumor cases. Especially intestinal type gastric carcinoma cells showed abundant positivity compared to diffuse type. Also some of the intestinal metaplastic cells adjacent to all of the gastric carcinoma showed positivity for TUNEL staining (Figure 2C). The mean apoptotic index in tumor cells was 0.70 ± 0.08, in intestinal metaplasias co-localizing to tumors it was 0.70 ± 0.03, and in intestinal metaplasias co-localizing to chronic gastritis was 0.09 ± 0.01 (Figures 1C and 2C). The difference between the mean apoptotic indexes in

Table 2 Results of TUNEL staining and immunohistochemical data

	Chronic gastritis co-localization of intestinal metaplasia	Adenocarcinoma co-localization of intestinal metaplasia	*P*
TUNEL (mean)	0.09 ± 0.01	0.70 ± 0.03	≤ 0.05
p53			
Positive	0	0	-
Negative	17	27	
BCL-2			
Positive	12	15	> 0.05
Negative	5	12	
BAX			
Positive	2	12	≤ 0.05
Negative	15	15	

intestinal metaplasias co-localizing to tumors and chronic gastritis was statistically significant ($P \leqslant 0.05$) (Table 2).

Immunohistochemically tumor cells were nuclear positive for p53 in 57.1% (24/42), cytoplasmic positive for bcl-2 in 47.6% (20/42) and again cytoplasmic positive for bax in 42.8% (18/42) in gastric carcinoma cases. There was no nuclear p53 positivity in areas of intestinal metaplasia adjacent to tumors or chronic gastritis. Intestinal metaplasia areas adjacent to tumors showed 55.5% (15/27) cytoplasmic bcl-2 positivity, whereas intestinal metaplasia areas adjacent to chronic gastritis showed 70.5% (12/17) cytoplasmic bcl-2 positivity (Table 2). Intestinal metaplasia areas adjacent to tumors showed 44.4% (12/27) cytoplasmic bax positivity, whereas intestinal metaplasia areas adjacent to chronic gastritis showed 11.7% (2/17) cytoplasmic bax positivity (Figures 1D and 2D). The difference in this comparison was statistically significant ($P \leqslant 0.05$) (Table 2).

DISCUSSION

This study demonstrated that TUNEL positivity of cells within the intestinal metaplasia areas adjacent to tumors is significantly higher than that of cells within the intestinal metaplasia areas adjacent to chronic gastritis. In addition, a significant increase in bax positivity of cells within the intestinal metaplasia areas adjacent to tumors in comparison to chronic gastritis suggested that the induction of apoptosis within these areas is at least partially bax mediated.

Correa have previously proposed a human model of gastric carcinogenesis for intestinal type gastric carcinoma, based on epidemiological, pathological, and clinical observations. According to this model, gastric carcinoma of the intestinal type originates in dysplastic epithelium, which in turn develops in atrophic gastritis followed by intestinal metaplasia. Experiments in several populations at high gastric cancer risk have documented a series of lesions, which are inflammation (chronic gastritis), atrophy, and loss of cellular differentiation. In reality the loss of differentiation appears to represent successive mutations, since the gastric epithelial cells disappear as such and are replaced by cells with intestinal phenotype, the daughters of these "mature" intestinal cells then display apparently progressive phenotypic changes, lose some of their normal cytoplasmic secretions and gain autonomy, which eventually leads to uninhibited replication and invasion of the neighboring tissues[1,11]. According to Correa, the normal gastric cells are replaced by cells expressing mature intestinal phenotype in the process of gastric carcinogenesis in humans and these are then replaced by cells with immature phenotypes capable of synthesizing the same cytoplasmic products of the neck cells. It is not clear if the neck cell acts as a stem cell or if it is derived from a precursor stem cell[1,12]. Supporting these suggestions our cases showed increased co-localization of intestinal metaplasia in intestinal type tumors in comparison to others.

Several classifications of intestinal metaplasia have been suggested by pathologists. Different observers proposed different definitions and criteria for classification[13]: Small intestine/colonic type[14,15], complete/incomplete type[16,17] and type I corresponds to the complete and types Ⅱ and Ⅲ to the incomplete type[18]. These classifications are based on only the intestinal properties. Recently a new classification was proposed based upon both gastric and intestinal differentiation status[13]: a gastric and intestinal mixed type, and solely intestinal type. In general intestinal metaplasia especially type Ⅲ-colonic metaplasia is suggested as a preneoplastic lesion in these classifications [1,19-21]. However, there are studies that do not support this hypothesis even for colonic type intestinal metaplasias[22]. Supporting this last idea, this study showed intestinal metaplasia areas co-localizing to both intestinal and diffuse type cancers. Although, the incomplete type of intestinal metaplastic is more commonly associated with an increased risk of malignant transformation[1,3,11], this study showed co-localization of type-I metaplasia to all chronic gastritis cases and in almost all, i.e., in all except 2, tumor cases. Those 2 tumor cases showed type-Ⅱ intestinal metaplasia co-localizing to tumors.

Studies investigating small gastric cancers showed that atrophy and dysplasia do not always co-localize with early neoplasia suggesting that metaplasia is not a definite preneoplastic lesion[23,24]. In addition, histogenesis studies of gastric cancers suggest that the majority of both diffuse and intestinal gastric cancers develop from non-metaplastic stem cells. It is believed that in these cases first gastric phenotype develops and intestinal phenotype is secondary to gastric phenotype. Thus, gastric cancer cells show heterogeneity in terms of gastric and intestinal phenotypic markers. However, it is not clear whether they consist of different types of cells or are derived from multipotential cells. All these findings suggest that the type of intestinal metaplasia is not directly important in the process of gastric cancer development[13].

Few studies investigating gastric cancer and involvement of apoptosis in this condition have shown the existence of apoptotic cells within the normal gastric mucosa, intestinalized glands, adenomatous dysplasia and carcinomas[25,26]. The involvement of proliferation and apoptosis in the transition from normal mucosa to atrophic gastritis and intestinal metaplasia has been studied especially in relation to HP related gastric carcinogenesis[26,27]. In normal gastric mucosa TUNEL

positive cells are abundant in foveolar epithelium, whereas in chronic gastritis this finding is accompanied by increased cell proliferation. In intestinal metaplasias adjacent to chronic gastritis, on the other hand, proliferation rate shows an increase whereas apoptotic index does not change significantly[26]. Findings of this study also support this last observation. However, TUNEL positivity in intestinal metaplasia areas adjacent to tumors was significantly higher than that of normal mucosa adjacent to tumors and to intestinal metaplasia adjacent to chronic gastritis.

Many *in vivo* and *in vitro* studies have also investigated the apoptotic process in gastric carcinomas and its relation to the p53 gene[28-31]. Findings from these studies are also in concordance with findings from this study in terms of observation of more apoptotic cells in intestinal type carcinomas in comparison to diffuse type carcinomas[4,25,28]. In one of these studies Ikeda *et al*[25] investigated TUNEL positivity based apoptotic index, ki-67 index and p53 expression in a series of minute, early and late stage gastric carcinomas. On the basis of well and poorly differentiated carcinoma classification they found asignificant increase in apoptotic and proliferating cells in well differentiated carcinomas in comparison to poorly differentiated carcinomas. However, p53 expression did not show any significant difference between minute, early and late stage gastric carcinomas. Based on these findings they suggested that gastric cancer development involves increased proliferation together with apoptotic mechanisms induced independently of p53 expression. However, there are also studies opposing this hypothesis[29,30,31]. Studies suggesting intestinal metaplasia as preneoplastic lesion have reported 30% p53 mutations especially in incomplete type metaplasias. p53 protein accumulation in gastric carcinogenesis, on the other hand, is first seen at the dysplasia stage[31]. Analysis of p53 expression in intestinal metaplasia areas adjacent to both gastric cancers and chronic gastritis on the basis of immunohistochemistry did not reveal any p53 protein accumulation in this study.

It has been shown that upregulation of bcl-2 expression in premalignant lesions is followed by its down regulation after malignant differentiation[31,32]. Analysis of bcl-2 expression in intestinal metaplasia areas adjacent to both gastric cancers and chronic gastritis on the basis of immunohistochemistry did not reveal any significant difference in this study. However, the comparison of bax expression between these two groups revealed increased bax expression in intestinal metaplasia areas adjacent to gastric cancer. The literature search revealed only a few studies, in which the investigators analyzed the expression patterns of apoptosis related proteins and their relation to preneoplastic lesions in gastric carcinoma[33-35]. However, none of these studies have investigated the relationship between the expression patterns of these apoptosis related proteins in intestinal metaplasia areas co-localizing to tumors. In this study, in addition to the above mentioned relationship the expression patterns of the apoptosis related proteins in intestinal metaplasia areas co-localizing to chronic gastritis was investigated. Finally, these expression patterns were compared to each other.

In conclusion, existence of apoptotic cells on the basis of TUNEL positivity is shown in intestinal metaplasias co-localizing to both diffuse and intestinal type gastric cancers in this study. Our results also suggested bax expression dependent induction of apoptosis especially in intestinal metaplasia areas adjacent to tumors. These findings strongly support the involvement of apoptotic mechanisms in the process of gastric carcinogenesis especially in the transition from intestinal metaplasia to gastric cancer. Finally, induction of apoptosis in intestinal metaplasia areas adjacent to tumors may involve different mechanisms than induction by chronic inflammation.

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