Fas and FasL Expression at Different Stages of Epithelial Malignant Transformation in the Large Intestine and It's Significance in Cancerous Apoptotic Evasion

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OBJECTIVE To explore the molecular mechanism of Fas counterattack by observing the expression of Fas and FasL and apoptosis of the epithelium and infiltrative lymphocytes at different stages of intestinal epithelia malignant transformations and further to analyze the relationships among these processes.

METHODS Using in-situ hybridization, the expression of Fas mRNA and FasL mRNA in paraffin-embedded tissues was determined. Apoptosis was assessed by the TUNEL method. The tumor specimens were as follows: 37 large intestinal adenocarcinomas, 26 malignant adenomas, 30 tubulo-villous adenomas, and 24 tubular adenomas. Six cases of non-tumor mucosas were used as controls.

RESULTS With the progression of malignant transformation, Fas mRNA expression increased slightly in benign adenomas, but significantly decreased in malignant diseases, but, FasL mRNA expression showed an increasing tendency. Apoptotic lymphocyte densities slso showed an increasing tendency. Apoptotic epithelial cell densities increased in benign tumors but decreased in malignant tumors. There was a positive correlation ($r=0.672$, $P<0.001$) between FasL expression and apoptotic lymphocyte densities in adenocarcinomas.

CONCLUSION With progression of large intestinal epithelial malignant transformation a progressive Fas counterattack develops. Cancer cells express FasL and induce tumor infiltrative lymphocytes to undergo apoptosis allowing the cancer to escape from immune surveillance through Fas-FasL interactions. In addition, the cancer cells may resist death signals transuded by FasL by down regulating Fas expression. This dual process expressed by immune effector cells and cancer cells, forms the Fas resistance mechanism-the prerequisites for Fas counterattack, resulting in inhibition of cancer cell apoptosis. Thus, we suggest that Fas counterattack, together with Fas resistance, both have a relationship to the formation and progression of large intestinal cancer.

KEYWORDS: large intestinal neoplasm, apoptosis, Fas mRNA, FasL mRNA, tumor infiltrative lymphocytes.

Apoptosis, also known as programmed cell death, is a genetically-controlled death-producing process. Fas and its ligand, FasL are transmembrane proteins of the tumor necrosis factor

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(TNF) family that are involved in apoptosis.[1] There is evidence demonstrating that apoptosis in large intestine cancers is inhibited, and that this inhibition is possibly associated with abnormalities in expression of certain apoptotic-regulating genes and with transduction of death signal within cells. The Fas/FasL system is an important cellular death receptor-ligand system. In 1996, O'Connell et al. [2] first raised the concept of "Fas counterattack". In this process, neoplastic cells express FasL, which induces infiltrative lymphocytes within tumor tissues to undergo apoptosis through Fas-FasL engagement, resulting in the lymphocytes losing their ability for immune surveillance. As a result tumor cells escape from Fas-mediated apoptotic induction through several of mechanisms.[3,4]

In our study, we used in-situ hybridization and the TUNEL technique to examine the regulation of Fas/FasL expression and the changes in apoptosis of local infiltrative lymphocytes during the progression of large intestinal epithelial malignant transformation. We initially explored the molecular mechanism of "Fas counterattack" and provide a theoretical basis for the prevention and treatment of this malignancy.

MATERIALS AND METHODS

Experimental tissues

We collected histologic specimens, which were surgically removed, fixed in formalin and embedded in paraffin. These tissues included 37 cases of large intestinal adenocarcinoma, 26 cases of cancer from adenomas, 30 cases of tubulovillous adenoma, and 24 cases of tubular adenoma from the General Hospital of Tianjin Medical University from January, 1988 to December, 1995. Controls consisted of 6 non-tumor large intestine mucosal tissues collected over the same period.

Experimental procedure

Experiment I

We examined the expression of Fas mRNA and FasL mRNA at different stages of large intestinal epithelial malignant transform by in-situ hybridization.

Main reagents

The assay kits for in-situ hybridization were purchased from Boshide Company, Wuhan. Reagent No. MK1113.

Main procedures

The sections were routinely deparaffinized step-wise, placed into 3% H_2O_2 for 10 min, and rinsed in distilled water. All of the following procedures were conducted at 37℃. Fresh pepsin digesting mixture in 3% citric acid was applied dropwise to the sections for 15 min. The sections were rinsed in 0.5 M PBS for 5 min for 3 times, rinsed once in distilled water followed by addition of 20 μl of in-situ hybridization fluid, covered with a special coverglass used for in-situ hybridization and hybridized over night. Then the sections were rinsed in 2× SSC for 5 min for 3 times, the inclusion reagent added dropwise for 30 min. Excess fluid was discarded, and rabbit anti-digoxin added dropwise followed by a 1h incubation. After rinsing the sections in 0.5M PBS for 2 min for 3 times and dropwise addition of the biotinized goat anti-rabbit IgG and incubation for 20 min, the sections were rinsed in 0.5 M PBS for 2 min for 3 times, SABC added dropwise for 20 min, rinsed in 0.5M PBS for 5 min for 3 times. Then visualize with DAB for 15 min, and rinse in water. After re-stain the nucleuses with the Mager hematoxylin, rinse in water.At last, the sections were dehydrated, hyalinized, and mounted.

Experiment II

We determined the degree of apoptosis of the tumor cells and infiltrative lymphocytes at different stages of large intestinal epithelial malignant transformation with the TUNEL technique.

Main reagents

Kits for apoptosis evaluation were purchased from the Baolingman Co., Germany.

Main procedures

The sections were deparaffinized with dimethylbenzene, and hydrated in graded alcohol. After removal of the intrinsic enzymes with 3% H_2O_2

in methyl alcohol, digestion with 10 μg/ml proteinase K, the sections were soaked in PBS for 5 min. The first antibody was added and the sections held at 37℃ for 2h.Then they were rinsed in PBS for 2 times for 5min. The sections were reacted in POD antibody for 1h, rinsed in PBS for 2 times for 5min and visualized in DAB for 15min. Then the nucleuses were re-stained with the hematoxylin. At last, the sections were dehydrated, hyalinized, and mounted.

Counting method of TUNEL-positive cells

The sections were stained through the procedures mentioned above and examined at a magnification of 400 times with a 256 lattice. The positive cells in the lattices were counted. Ten microscopic fields were counted per section per group. The mean value served as the apoptotic cellular density of that lesion.

Statistical method

SPSS software was used to analyze the density of positive cells for each group and the relation between the density of cells with FasL mRNA positive expression with that of the locally infiltrated lymphocytes in the apoptotic region in malignant lesions was analyzed by analysis of variance and the interclass correlations.

RESULTS

Morphological features of Fas and FasL mRNA expression, and apoptotic cells at different stages of large intestinal epithelial malignant transformation

Fas and FasL mRNA positive material mainly lays in the cytoplasm of large intestinal epithelial cells and tumor cells, exhibiting a brown-yellow color (visualized with DAB). The TUNEL positive material was found in the nucleus, and also exhibited a brown-yellow color (visualized with DAB). The distribution of positive material in the tissues at the different stages of malignant transformation of large intestinal epithelium was found as follows.

Non-tumor intestinal mucosa

The Fas and FasL mRNA positive cells all lay in the superficial-layer epithelium, and were distributed in a cluster shape. The locally infiltrated apoptotic lymphocytes scattered.

Tubular adenoma and tubulovillous adenoma

The positive cells which expressed Fas and FasL mRNA few in number, distributed from the superficial-layer of mucosa to the bottom-layer, which were scattered or had a tendency of cluster-distribution. The TUNEL-positive cells increased slightly as compared with non-tumor intestinal mucosa, and were mostly distributed in a cluster shape.

Non-malignant transformed area of adenoma

The expression of Fas mRNA decreased slightly as compared with tubular adenoma and tubulovillous adenoma. And those positive for FasL mRNA increased slightly as compared with tubular adenoma and tubulovillous adenoma, and were scattered or had a tendency of a cluster-distribution. The TUNEL-positive cells were increased markedly as compared with tubular adenoma and tubulovillous adenoma, which were scattered or had a tendency of a cluster-distribution.

Malignant adenoma and adenocarcinoma

The expression of Fas mRNA decreased significantly as compared with the non-malignant transformed area of adenoma, and those cells positive for FasL mRNA increased significantly as compared with the non-malignant transformed area of adenoma, and were distributed in a scatter-shape or sheet-shape. The TUNEL-positive lymphocytes of these lesions decreased to a larger extent than that found in adenomas. The locally infiltrated lymphocytes showed an increased apoptotic tendency, and were mostly distributed in a scatter-shape.

Determination of experimental indexes at different stages of large intestinal epithelial malignant transformation and their correlations

Table 1 shows the comparison of the density of Fas mRNA positive cells at different stages of large

intestinal epithelial malignant transformation.

The degree of Fas mRNA expression was determined as follows: The expression in normal non-tumor mucosa was at a moderate level, whereas that in benign adenomas was slightly increased(Fig.1). Areas of non-malignant transformed adenoma showed a lowered trend of expression, and that of malignant lesions a significant depression of expression. There were significant differences between the latter and those of the non-malignant transformed area of adenomas. Adenocarcinoma showed the lowest level of Fas mRNA expression. The differences between the expression in adenocarcinoma and that in the malignant transformed area of adenoma were of statistical significance (P=0.003)(Table 1).

Comparison of the density of FasL mRNA positive cells at different stages of malignant transformation of the large intestinal epithelium

The degree of expression of FasL mRNA showed a progressive increased trend during the course of malignant transformation of the large intestinal epithelium, whereas that of the malignant lesions increased noticeably (Table 2). The density was the highest in adenocarcinomas, but the differences between its level of expression and that of the malignant transformed areas of adenomas were not of statistical significance (P=0.977).

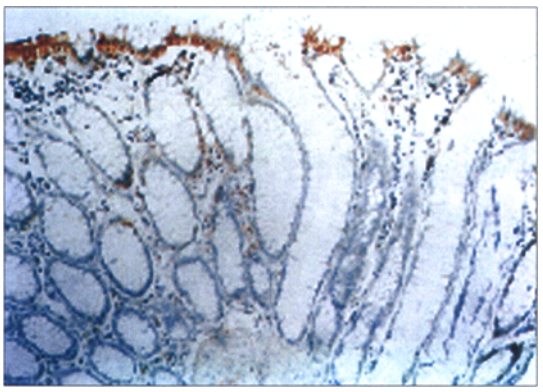

Fig.1. The Fas mRNA expression was relatively higher in the large intestinal tubular adenoma, showing a cluster distribution pattern, Fas mRNA× 100.

Comparison of the density of apoptotic epithelial cells at the different stages of malignant transformation of the large intestinal epithelium and its relation to Fas mRNA positive cells

The number of apoptotic epithelial cells in benign

Table 1. Comparison of the density of Fas mRNA positive cells at different stages of malignant transformation of the large intestinal epithelium ($\bar{x}\pm s$)

				Statistical analyses	
Group	n	Density	Square root of density	Contrast groups	*P*
①Non-tumor mucosa	5	39.60 ± 6.51	2.50 ± 0.11	①versus④	0.047
②Tubular adenoma	22	50.93 ± 21.64	2.65 ± 0.31	①versus⑤	<0.001
③Tubulovillous adenoma	29	45.91 ± 24.51	2.52 ± 0.37	①versus⑥	<0.001
④Non-malignant area*	24	25.47 ± 14.76	2.18 ± 0.32	②versus④	<0.001
⑤Malignant area*	24	11.92 ± 9.47	1.78 ± 0.35	②versus⑤	<0.001
⑥Adenocarcioma	35	5.88 ± 5.10	1.51 ± 0.30	②versus⑥	<0.001
				③versus④	<0.001
				③versus⑤	<0.001
				③versus⑥	<0.001
				④versus⑤	<0.001
				④versus⑥	<0.001
				⑤versus⑥	0.003
F			48.959		<0.001

Note: The groups marked with "*" refer to the adenoma in this and following tables.

Table 2. Comparison of the density of FasL mRNA positive cells at different stages of malignant transformation of the large intestinal epithelium ($\bar{x}\pm s$)

Group	n	Density	Square root of density	Statistical analyses	
				Contrast groups	*P*
①Non-tumor mucosa	5	8.94 ± 7.43	0.83 ± 0.37	①versus②	0.023
②Tubular adenoma	23	19.85 ± 12.25	1.19 ± 0.34	①versus③	0.024
③Tubulovillous adenoma	28	22.28 ± 14.63	1.19 ± 0.37	①versus④	0.003
④Non-malignant area*	24	26.12 ± 17.14	1.32 ± 0.33	①versus⑤	<0.001
⑤Malignant area*	24	50.34 ± 22.74	1.64 ± 0.26	①versus⑥	<0.001
⑥Adenocarcioma	37	52.01 ± 27.60	1.64 ± 0.29	②versus⑤	<0.001
				②versus⑥	<0.001
				③versus⑤	<0.001
				③versus⑥	<0.001
				④versus⑤	<0.001
				④versus⑥	<0.001
F			14.552		<0.001

adenomas was relatively high (Fig.2), and that in the non-malignant transformed areas of adenomas showed the highest level (Table 3) which was highly significant. That of the non-tumor mucosa was the lowest with a progressively decreasing tendency during the course of adenoma-malignant transformation of adenoma to adenocarcinoma. There was a positive correlation between its density and that of apoptotic epithelial cells (r=0.190, $P<0.05$).

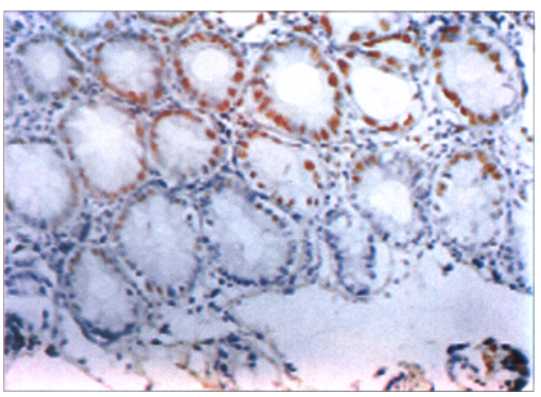

Fig.2. The density of the apoptotic cell in the epithelium of the large intestinal tubular adenoma was relatively higher, TUNEL× 200.

Comparison of the density of apoptotic, locally infiltrated lymphocytes at different stages of malignant transformation of the large intestinal epithelium and its correlation with FasL mRNA positive cells in malignant lesions

The change in the density of apoptotic, locally infiltrated lymphocytes in benign lesions was not significant as all were relatively low. However, apoptotic lymphocytes in the malignant lesions showed an increased tendency. Areas of malignant transformation of adenomas were the highest, but the differences between the latter and that of adenocarcinomas were not statistically significant ($P=0.380$) (Table 4). There was a positive correlation between the density of apoptotic, locally infiltrated lymphocyte and that of FasL mRNA positive cells in the adenocarcinoma cases (r=0.672, $P<0.001$). However, there was no correlation between apoptotic lymphocytes and FasL mRNA expression in malignant transformed areas of adenomas (r=0.153, $P>0.05$).

DISCUSSION

Fas, a type I transmembrane protein [5] belongs to the receptor family for the tumor necrosis factor (TNF) and nerve growth factor (NGF). Fas is characterized by a cysteine-rich extracellular domain and a highly

Table 3. Comparison of the density of apoptotic epithelial cells at different stages of the malignant transformation in the large intestinal epithelium ($\bar{x}\pm s$)

Group	n	Density	Square root of density	Statistical analyses	
				Contrast groups	*P*
①Non-tumor mucosa	6	15.02 ± 11.14	3.62 ± 1.53	①versus②	<0.001
②Tubular adenoma	24	46.31 ± 18.86	6.44 ± 1.82	①versus③	0.02
③Tubulovillous adenoma	30	29.43 ± 16.66	5.20 ± 1.45	①versus④	<0.001
④Non-malignant area*	23	68.42 ± 19.61	8.19 ± 1.20	②versus③	0.003
⑤Malignant area*	23	22.01 ± 12.07	4.51 ± 1.34	②versus④	<0.001
⑥Adenocarcioma	37	18.64 ± 12.88	4.03 ± 1.56	②versus⑤	<0.001
				②versus⑥	<0.001
				③versus④	<0.001
				③versus⑥	0.002
				④versus⑤	<0.001
				④versus⑥	<0.001
F			27.852		<0.001

conserved cytoplasmic region of 70-80 amino acids known as the death domain (DD), which has been shown to be both necessary and sufficient for transduction of an apoptotic signal.[1]

In this article, using in-situ hybridization, we observed the trait of FasL mRNA expression at different stages of progression from the non-tumor mucosa, adenoma, malignant transformed adenoma, to adenocarcinoma of the large intestine. We also confirmed that FasL mRNA expression showed a progressive increase associated with clinical development during the course of malignant intestinal epithelium transformation. The process of FasL mRNA positive expression in malignant lesions was significantly higher compared to benign lesions. These results suggest that the basis of the Fas counterattack mechanism lay in the intestinal carcinoma. In addition, the trait of the FasL mRNA expression indicated that FasL expression and the FasL related immune escape appeared at an early stage in the malignant transformation. The density of apoptotic infiltrative lymphocyte increased in the malignantly transformed area of adenoma and in the adenocarcinoma, suggesting that there was an active apoptotic deletion of lymphocytes in the malignant lesion, leading to downregulation of the lymphocytes. Thus, immune invasion is ineffective in inhibiting the development of the cancer.[6-9]

The resistance of tumor cells to apoptosis mediated by Fas is an important prerequisite for Fas counterattack. If Fas and FasL are expressed in the same cancer cell, autocrinal "suicide" will occur. And if Fas and FasL are expressed in the adjacent cells respectively, the paracrinal "fratricide" will occur. In our study, of the malignant transformation, Fas mRNA expression exhibited a tendency toward a significant decrease after a slight increase. The decrease was significant in the malignant lesions, suggesting that Fas resistance does exist in the malignant lesions of the large intestine, through downregulation and deletion of Fas.[10-12]

Therefore, we can deduce that during the progressive course of development from adenoma to adenocarcinoma, the tissues increase apoptosis by some factors, such as the Fas upregulation resulting in inhibition of cancer formation. But because FasL expression increases progressively, Fas counterattack gradually enhances so, malignant transformation continues to advance, and the cancer-cell death decreases furthered by Fas resistance, since the cancer cells with the dual features of Fas counterattack and Fas resistance selectively dominate, tumor growth is

enhanced. As a result, this processes not only causes the formation of the intestinal carcinoma, but also enhances the malignant phenotype. The modulation of resistance in Fas-mediated cytotoxicity and apoptosis of bladder cancer cells has been demonstrated by Mizutani et al. [13]

In summary, elimination of Fas counterattack and Fas resistance mechanisms in cancers by using different methods, such as drugs and the biotherapy, is becoming a powerful measure to treat large intestinal cancers which employ the Fas system.

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