Interaction between COX-2 and iNOS in Gastric Carcinoma

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Abstract **Objective** To evaluate the interaction between cyclooxygenase(COX-2) and inducible nitric oxide synthase (iNOS) in gastric adenocarcinoma. **Methods** Immuno-histochemical stain was used for detecting the expression of COX-2 and iNOS in 45 resected specimens of gastric adenocarcinoma; the monoclonal antibody against CD34 was used for displaying vascular endothelial cells, and microvascular density (MVD) was detected by counting of CD34-positive vascular endothelia cells. Paracancerous tissues were examined as control. **Results** Immunohistological staining with COX-2 or iNOS-specific polyclonal antibody showed cytoplasmic staining in the cancer cells. The rate of expression COX-2, iNOS and MVD index in gastric cancers were significantly increased, compared with those in the paracancerous tissues (77.78% vs 33.33%, 68.89% vs 17.78%, 58.13±19.99 vs 24.02±10.28, $P<0.01$, $P<0.005$, $P<0.05$, respectively). Moreover, MVD in COX-2-or iNOS-positive specimens was higher than that in COX-2-or iNOS-negative specimens (61.29±14.31 vs 45.38±12.42,54.68±16.07 vs 31.42±12.34, $P<0.05$, $P<0.001$).Both COX-2 and iNOS expression were positively correlated with MVD (r=0.63, r=0.54,$P<0.05$, $P<0.05$). The rate of COX-2 expression in the iNOS-positive was significantly increased, compared with that of the negative ($P<0.05$). **Conclusion** Expression of COX-2 and iNOS might cooperatively produce the occurance of gastric carcinoma and involve in tumor angiogenesis in gastric carcinoma, both COX-2 and iNOS may be new therapeutic target for anti-carcinogenesis.

Key words Gastric adenocarcinoma; Cyclooxygenase; Nitric oxide synthase; Immunohistochemistry

Cyclooxygenase (COX) is a key enzyme in the conversion of arachidonic acid to prostaglandin, and two isoforms of COX, namely COX -1 and COX-2, have been identified [1,2]. COX-1 is constitutively expressed in many tissues and is considered to be involved in various physiological functions, whereas COX-2 is induced by pathological stimuli, such as inflammation,various growth factors and cytokines produced by tumor cells [1-3]. Nitric oxide synthase (NOS) also exists in constitutive and inducible form[4]. The constitutive form results in the production of physiologic levels of NO that are important in many tissues, whereas iNOS is related to a high output pathway and is responsible for various pathological processes[4].

Elevated levels of NO produced by expression of iNOS and high levels of prostaglandins generated by COX-2 are important mediators of inflammatory responses. Recently, it has been recognized that both COX-2 and iNOS play important roles in the human carcinogenesis[5-8].We have also reported that expression of COX-2 and iNOS is responsible for enhanced tumor growth and metastasis in gastric carcinoma[9,10].

Recent data indicated that NO produced by iNOS increases the activity of COX-2 [11].We investigated the coexpression of COX-2, iNOS and microvascular density (MVD) in gastric cancer. The aim of this study was to determine the interection between COX-2 and iNOS in gastric carcinoma.

MATERIALS AND METHODS

Materials

Forty -five patients with gastric adenocarcinomas who were confirmed pathologically received gastroectomy in our hopital from January 2000 to October

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2001. In this subjects, gastric tumors and paracancerous tissues (more than 5cm away from the focus) were obtained from resected specimen. Of these, 35 patients were male, and 10 female, with a mean age of 57.51± 10.73(33 to 78)years old. Patients who had received radiotherapy or chemotherapy before gastroectomy were excluded. Histologically, they were classified by the WHO criteria,5 were highly differentiated adenocarcinoma, 10 moderately−differentiated, 27 poorly−differentiated, and 3 undifferentiated. As regards to the size of cancer, 20 were <5cm, 25≥5cm; 33 tumors invaded to the serosa and 12 tumors did not. 36 cases had local lymph node metastasis.

Reagents and methods

Antibody against COX−2 was purchased from Santa Cruz Biotechnology Inc; antiboby against iNOS was purchased from Wu Han Boster Co.Ltd; antiboby against CD34 and ready to use SP immunohistochemical reagent box were purchased from Fujian Maixin CO, Ltd. Formalin−fixed, paraffin−embedded surgical specimens from 45 cases of gastric carcinoma were available and sliced sequentially with a thickness of 4μm. The slices carrying the detected antigen were dyed with SP immunohistochemical staining method, and those in the control group were dyed according to the above method, with the first antibody substituted by PBS.

Statistical methods

The data were presented as $\chi\pm s$; numerical variable by χ^2 test; enumeration data by $t-$ test; COX−2, iNOS relationship with MVD by spearman rank correlation test(depending on the quantitative index of COX−2, iNOS and MVD).

RESULTS

The cytoplasm of the gastric cancer cells stained brown granules were identified to be positive for COX−2 or iNOS. Only the nucleuses staining blue were identified to be negative for COX−2 or iNOS. COX−2 and iNOS expression were scored semi−quantitatively respectively according to the density and the percentage of positively stained tumor cells into score 0,1,2,3. A minimum of 10 high power view was used to assess COX−2 or iNOS expression level. If the sum of two scores was 1−3, the slice would be considered as low−expression of COX−2 or iNOS; whereas 4−6, it would be considered as high−expression of COX−2 or iNOS. When the cytoplasm of theirs stained brown or brownish yellow,vascular endothelial cells were CD34−positive; the microvessels were counted according to the number of single endothelial cell or endothelial cell clustor showing brownish yellow granules in the cytoplasm. The slices were observed first microscopically under the low power (×40), then selected the most dense area of microvessel and the high power (×200, the surface area of every vision field being 0.785mm2), and the number of microvessel in 3 vision field were counted and took the average as MVD of this specimen[12].

COX−2, iNOS and MVD expression and distribution

The positive expression of COX−2 and iNOS was mainly diffusedly located at in the cytoplasm. 77.78% (35/45)cases of gastric carcinomas showed COX−2 positive expression while high−expression was detected in 22 cases, low−expression in 13 cases. 33.33%(15/45)cases of paracancerous tissues showed COX−2 positive expression while high−expression was only detected in 3 cases. The rate and density of COX−2 expression in cancerous tissues were significantly higher than that in paracancerous tissues (χ^2=18, χ^2=6.09, P<0.005, P<0.05, respectively). The positive expression rate of iNOS was 68.89%(31/45), while high−expression was detected in 24 cases, low−expression in 7 cases. 17.78%(8/45) of paracancerous tissues showed iNOS positive expression, while high−expression was not detected. The rate and density of iNOS expression in cancerous tissues were significantly higher than that in paracancerous tissues (χ^2=23.94, P<0.005).

The mean MVD in gastric carcinoma was significantly higher than that in para−cancerous tissues(58.13± 19.99,24.02±10.28,t=10.18, P<0.001).The positive expression of CD34 was mainly presented as brownish yellow or brownish granules in the cytoplasm of vascular endothelial cells. New blood vessles in the cancerous lesions had no regular figure and were not well dis-

Table The relationship between the rate of COX−2 and iNOS expression

COX−2	iNOS		n
	positive	negitive	
positive	28	7	35
negitive	3	7	10
n	31	14	45

χ^2=6.93, P<0.05, the rate of COX−2 expression in the iNOS−positive compared with the negative.

tributed.

The relationship between the rate of COX−2 and iNOS expression

There was significant correlation between the expression of COX−2 and iNOS in gastric carcinoma, as shown in table.

The relationship between the rate of COX−2, iNOS expression and MVD

The result showed that MVD (61.29±14.31)in the COX−2 positive gastric cancerous tissues was higher than that(45.38±12.43)in the COX−2 negative one(t=5.64, P<0.001).The expression of COX−2 was positively correlated with MVD (r=0.63, P<0.05). MVD (54.68±16.07)in the iNOS−positive gastric cancerous tissues was higher than that (31.42±12.34)in the negative one (t=7.70, P<0.001). The expression of iNOS was positively correlated with MVD (r=0.54, P<0.05).

DISCUSSION

In the current study, we found that the rate of COX−2 expression in gastric cancer was significantly increased, compared with that in the paracancerous tissues, the expression of COX−2 showed cytoplasmic staining. A similar pattern of COX−2 expression has previously been found in human gastric cancer [13−17]. Human gastric mucosa normally expresses barely detectable levels of COX−2 protein [18,19].The above data demonstrated COX−2 was up−regulated in human gastric cancer, which suggested that COX−2 may play an important role in occurrence of gastric cancer. We found that the MVD in COX−2 positive tumors was significantly higher than that in COX−2 negative tumors, MVD in gastric carcinoma was higher than that in paracancerous tissues, and its distribution was similar to the pattern of COX−2 in gastric carcinoma. A close correlation was detected between MVD and COX−2 (P<0.01), which indicated that COX−2 was closely related to tumor angiogenesis, and may be one of important factors involved in gastric carcinoma angiogenesis.

It is shown that the expression of iNOS in most tumor tissue is higher than that in the normal one [20,21]. The carcinogenesis of iNOS is mainly caused by its product NO and the metabolites of NO, such as activated oxygen, activated nitrogen and excessively oxidated nitrite, which can cause the damage of DNA, induce mutation and increase oxidative stress [22,23]. Excessive iNOS may restrain apoptosis of tumor cells [7]. In this study, we found that the rate of iNOS expression in gastric cancer was significantly increased, compared with that in the paracancerous tissues, a close correlation was present between MVD and iNOS(P<0.01). The above findings suggusted iNOS was closely related to the formation of gastric carcinoma, and may be one of important factors involved in gastric carcinoma angiogenesis.

COX−2 shares significant features with iNOS in terms of its tissue distribution and participation in pathophysiological phenomena, iNOS and COX−2 work cooperatively and increases its catalytic output[24−30]. In vitro, on adding the NO donor, the PGE2 level was increased 2~20 times due to increased COX−2 expression. This increase of COX−2 expression by SNAP or PMA (potent inducer of both iNOS and COX−2) was blocked to various degrees by NO scavengers and NOS inhibitors [31]. Although there is extensive evidence that NO increases COX−2 activity, the mechanisms of this effect have not been elucidated. Liu *et al* reported that NO through catenin signaling stimulated PEA3, an Ets transcription factor, to increase COX−2 activity[32].

In this study, we demonstrated that the expression level of COX−2 and iNOS was significantly higher in gastric cancer tissue than in the paracancerous tissues, and there was significant correlation between the expression of COX−2 and iNOS in carcinoma tissues.

The expression of COX−2 and iNOS may be one of the factors that contribute to gastric carcinogenesis. Moreover, both the expression COX−2 and iNOS had a strong correlation with the index of MVD in gastric cancer. Similarly, in the study of rat lung carcinogenesis, COX−2 and iNOS expressions were elevated simultaneously, and associated with MVD[33]. These above data indicated that COX−2 and iNOS may cooperatively produce angiogenesis, which is one of the mechanisms COX−2 and iNOS involving in carcinogenesis.

In conclusion,both COX−2 and iNOS expression in gastric carcinoma were higher than that in the paracancerous tissues, and were closely related to MVD. There was significant correlation between positive COX−2 and positive iNOS expression.These findings suggested COX−2 and iNOS expression might cooperatively contribute to the occurance of gastric carcinoma and involve in tumor angiogenesis in gastric carcinoma, both COX−2 and iNOS may be new therapeutic target for anti−carcinogenesis.

REFERENCES

1. Williams CS, DuBois RN. Prostaglandin endoperoxide synthase: why two isoforms? Am J Physiol, 1996, 270: 393−400.
2. Eberhart CE, Dubois RN. Eicosanoids and the gastrointestinal. tract.Gastroenterology, 1995,109: 285−301.
3. DuBois RN, Awad J, Morrow J, *et al.* Regulation of eicosanoid production and mitogenesis in rat intestinal epithelial cells by tranasforming growth factor−alpha and phorbol ester. J Clin Inves,1994, 93:493−498.
4. Michel T,Feron O. Perspective series: nitric oxide and nitric oxide synthases. J Clin Investig, 1997, 100:2146−2152.
5. Xue YW, Zhang QF, Zhu ZB, *et al.* Expression of cyclooxygenase−2 and cliniopathologic features in human gastric adenocarcinoma.World J Gastroenterol, 2003, 9:250−253.
6. Qiu DK, Ma X, Peng YS, *et al.* Significance of cyclooxygenase−2 expression in human primary hepatocellular carcinoma. World J Gastroenterol 2002, 8: 815−817[PMID: 12378621].
7. Kong G, Kim EK, Kim WS, *et al.* Role of cyclooxygenase−2 and inducible nitric oxide synthase in pancreatic cancer. J Gastroenterol Hepatol, 2002, 17: 914−921.
8. Hong SK, Gul YA, Ithnin H, *et al.* Expression of beta−catenin, COX−2 and iNOS in colorectal cancer:relevance of COX−2 and iNOS inhibitors for treatment in Malaysia. Asian J Surg, 2004, 27:10−17.
9 . Song ZJ, Gong p, Wu YE. Relationship between the expression of iNOS,VEGF, tumor angiogenesis and gastric cancer. World J Gastroenterol, 2002, 8:591−595.
10. Li HX, Chang XM, Song ZJ, *et al.* Correlation between expression of cyclooxygenase−2 and angiogenesis in human gastric adenocarcinoma. World J Gastroenterol, 2003, 9: 674−677.
11 .Perez−Sala D, Lamas S. Regulation of cyclooxygenase−2 expression by nitric oxide in cells. Antioxid Redox Signal. 2001, 3: 231−248.
12. Weidner N, Folkman J, Pozza F, *et al.* Tumor anginogenesis:a new significant and independent prognostic indicator in early state breast carcinoma. J Natl Cancer Inst .1992, 84: 1875−1887.
13. Uefujik K, Ichikura T, Mochizukia H, *et al.* Expression of cyclooxygenase−2 protein in gastric adenocarcinoma. J Surg Oncol, 1998, 69: 168−172.
14. Ratnasinghe D, Tangrea JA, Roth MJ, *et al.* Expression of cyclooxygenase−2 in human adenocarcinomas of the gastric cardia and corpus. Oncel Rep, 1999, 6: 965−968.
15. Lim HY, Joo HJ, Choi JH, *et al.* Increased expression of cyclooxygenase−2 protein in human gastric carcinoma.Chin Cancer Res, 2000, 6: 519−525.
16. Van Rees BP, Saukkonen K, Ristimaki A, *et al.* Cyclooxygenase−2 expression during carcinogenesis in the human stomach. J Pathol, 2002, 196: 171−179.
17. Ohno R, Yoshinaga K, Fujita T, *et al.* Depth of invasion parallels increased cyclooxygenase−2 levels in patents with gastric carcinoma. Cancer, 2001, 91: 1876−1881.
18. Mizuno H, Sakamoto C, Matsuda K, *et al.* Induction of cyclooxygenase 2 in gastric mucosal lesions and its inhibition by the specific antagonigt delays healing in mice.Gastroenterology, 1997, 112: 387−397.
19. Kargman S, Charleson S, Cartwright M, *et al.* Characterization of prostaglandin G/H synthase 1 and 2 in rat, dog, monkey and human gastrointestinal tracts.Gastroenterology, 1996, 111: 445−454.
20. Yagihashi N, Kasajima H, Sugai S, *et al.* Increased in situ expression of nitric oxide synthase in human colorectal cancer. Virch Arch, 2000, 436: 109−114.
21. Kojima M, Morisaki T, Tsukahara Y, *et al.* Nitric oxide synthase expression and nitric oxide production in human colon carcinoma tissue. J Surg Oncol, 1999, 70: 222−229.
22. Sandhu JK, Privora HF, Wenckebach G, *et al.* Neutrophils, nitric−oxide synthase,mutations in the mutatect murine tumor model. Am J Pathol, 2000, 156: 509−518.
23. Chen J, Xie Y, Zhong XJ, *et al.* Significance of COX−2 and iNOS expression in colorectal adenocarcinoma. China Journal of Modern Medicine, 2004, 14: 1−4.
24. Perkins DJ, Kniss DA. Blockade of nitric oxide formation

down−regulates cyclooxygenase−2 and decrease PGE2 biosynthesis in macrophages. J Leukoc Biol, 1999, 65: 792−799.

25. Watkins DN,Garlepp MJ, Thompson PJ. Regulation of the inducible cyclooxygenase pathway in human cul tured airway epithelial (A549) cells by nitric oxide. Br J Pharmacol, 1997, 121: 1482−1488.

26. Watanabe K,Kawamori T, Nakatsugi S, *et al.* COX−2 and Inos, good targets for chemoprevention of colon cancer. Biofactors, 2000, 12: 129−133.

27. Chiarugi V, Magnelli L, Gallo O. COX−2, iNOS and p53 as play makers of tumor angiogenesis. Int J Mol Med, 1998, 2: 715−719.

28. Li H, Chen F, Yu L, *et al.* Expression of inflammation related enzymes during experimental rat lung carcinogenesis. Zhonghua Zhong Liu Za Zhi, 2002, 24:316−319.

29. Liu Q, Chan ST, Mahendran, Ratha. Nitric oxide induces cyclooxygenase expression and inhibits cell growth in colon cancer cell lines. Carcinogenesis, 2003, 24: 637−642.

30. Gallo O, Schiavone N, Papucci L, *et al.* Down−regulation of nitric oxide synthase−2 and cyclooxygenase−2 pathway by p53 in squamous cell carcinoma. Am J Pathol 2003, 163: 723−732.

31. Park SW, Lee SG, Song SH, *et al.* The effect of nitric oxide on cyclooxygenase−2 (COX−2) overexpression in head and neck cancer cell lines. Int Cancer, 2003, 107: 729−738.

32. Liu Y, Borchert GL, Phang JM. PEA3, an Ets transcription factor, mediates the induction of cyclooxygenase−2 by nitric oxide in colorectal cancer cells. J Biol Chem, 2004,19: [Epub ahead of print].

33. Li HG, Chen FC, Liu MQ, *et al.* Cyclooxygenase−2,inducible nitric oxide synthase protein expression and MVD during experimental rat lung carcinogenesis. Chin J Lung Cancer, 2003, 6: 102−106.