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A Study on the Expression of EGFR and c-erbB-2 and Nuclear DNA Content in the Stomach Cancer Tissue

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Abstract: The expression of EGFR and c-erbB-2 as well as the measurement of nuclear DNA content was evaluated in stomach cancer tissues. In addition, changes in the expression of EGFR in stomach cancer cells in mixed culture with lymphocytes were also studied by flowcytometry. The frequencies of expression of EGFR and c-erb B-2 and of the aneuploid pattern in analysis of nuclear DNA content were 35.5% and 59.3%, in the primary focus respectively. By histological types, the rates of positive c-erbB-2 expression and the appearance of aneuploidy were high in differentiated cancer, while the EGFR expression rate showed no difference according to histological types. As to lymph node metastasis, the involvement of the lymph nodes in EGFR-positive cases was more significant than that in EGFR-negative viz. 86.4% vs. 43.2%. On the other hand, the attitude of EGFR expression was clearly altered when stomach cancer cells were incubated with lymphocytes. It is possible to draw the conclusion that the weaker the expression of EGFR in stomach cancer cells, the greater the susceptibility to lymphocytes in gastric cancer cells.

Introduction

Recently attention has been focused on the relation between cell proliferation and malignant transformation in association with oncogenes. There are reports, concerning the study on the relationship between the expression of the epidermal growth factor receptor (EGFR), and the c-erbB oncogene, and the frequency of the occurrence of metastases including the prognosis in breast cancer[1] and bladder cancer.[2] As to the c-erbB-2 protein, which is structurally analogous to EGFR, recent studies focus on the relationship with its metastasis and prognosis, particularly in cases of breast cancer.[3,4] There also is a report on the interaction between EGFR and c-erbB-2.[5] In the present study, the expression of EGFR and c-erbB-2 protooncogenes in stomach cancer was immunohistologically investigated and compared with the nuclear DNA content which is generally used as an indicator of biologically malignant behavior. In addition, for the purpose of clarifying the change in the expression of EGFR by lymph node metastasis, the EGFR expression was investigated in the mixed culture with stomach cancer cells and lymphocytes.

Subjects and Methods

Sixty-two stomach cancer specimens excised in this Department during the period of January 1989 to January 1991 were used.

1. Immunostaining

Using a cryostat, 3 consecutive sections, each 5 *um* thick, were cut out from each frozen tissue and one section was used for EGFR, another for c-erbB-2, and the remainder for H-E staining. Each section was mounted on slide glass, air-dried and fixed with acetone for 5 min. The section was then washed with PBS (phosphate buffered saline) for 5 min, 3 times and stained by the ABC method. Thus, after the endogenous peroxidase activity was inhibited with 3% H_2O_2, normal sheep serum (NSS) was reacted at room temperature for 20 min. and, then, anti-EGFR antibody (OSI) or anti-c-neu antibody (OSI) at 4 ℃ overnight. After washing with PBS, biotylated anti-mouse IgG was reacted at room temperature for 30 min. The section was then washed with PBS, treated with ABC reagent (Vector lob) and reacted with diaminobenzidine for 5 min. The section was then counter-stained with hematoxylin, mounted and examined under the microscope.

2. Determination of nuclear DNA content

The tissue was cut into pieces with ophthalmological acissors, denuded with 0.1% Triton X and stained with 100 *ug*/ml propidium iodide and the nuclear DNA content was determined with the FACScan (Becton-Dikinson).

3. Change in EGFR expression in a stomach cancer cell-lymphocyte mixed culture system

As the stomach cancer cell, MKN-28, a gastric tubular adenocarcinoma cell line, was used. From the peripheral blood of normal subjects and the metastasis-negative lymph nodes of the 6 stomach cancer patients, lymphocytes were separated by the method of Ficoll-Conray. MKN-28 was sown on a 100 mm dish and, after 24 hours, the lympho-

cytes were added. After 24, 48 and 72 hours, the cells were harvested with 1/2 TE and counted. After washing with 0.1% BSA/PBS, FITC-labelled anti-mouse IgG antibody was reacted at 4 ℃ for 45 min. and the change in FITC fluorescence was determined with the FACScan. Statistical analysis was made using X^2 test and Student-t test.

Results

1. Expression of EGFR and c-erbB-2 and nuclear DNA content by the depth of invasion (Table 1)

When the depth of invasion was classified into m, sm and pm or below, whereas the frequency of EGFR aneuploidy was high at pm or below, that of c-erbB-2 was rather high, as a tendency, in the early stage of cancer, although there was no significant difference.

Table 1. Expression of EGFR and c-erbB-2 and nuclear DNA content by depth of invasion

	EGFR	c-erbB2	Aneuploidy
m. sm	5/21 (23.8%)	9/21 (42.9%)	7/17 (41.2%)
pm~	17/41 (41.5%)	11/41 (26.8%)	25/ 3 (64.1%)
Total	22/62 (35.5%)	20/62 (23.8%)	32/54 (59.3%)

2. Frequency by histological type (Table 2)

When the cancer was classified into the differentiated and undifferentiated, there was no difference in the expression of EGFR between the two histological types. The frequency of aneuploidy was high in differentiated cancer and c-erbB-2 also showed a high frequency of expression in differentiated cancer ($p < 0.05$), viz. 14/29 (48.3%) for differentiated cancer vs. 6/33 (18.2%) for undifferentiated cancer.

Table 2. Expression of EGFR and c-erbB-2 and nuclear DNA content according to histologic type

	EGFR	c-erbB2	Aneuploidy
diff.	10/29 (34.5%)	14/29 (48.3%) *	18/25 (72.0%)
undiff.	12/33 (36.4%)	6/33 (18.2%) *	14/29 (48.3%)
Total	22/62 (35.5%)	20/62 (32.3%)	32/54 (59.3%)

* $p < 0.05$

3. Relation between EGFR or c-erb-B-2 and DNA ploidy (Table 3)

The frequency of aneuploidy in EGFR-positive cases was 12/18 (66.7%) and that in EGFR-negative cases was 20/36 (55.6%). On the other hand, the frequency of aneuploidy in c-erbB-2-positive cases was 12/19 (63.2%) and that in c-erbB-2-negative cases was 20/35 (57.1%). Thus, for both of EGFR and c-erbB-2, the frequency of aneuploidy tended to be slightly higher in positive cases.

Table 3. Relation between EGFR or c-erb-B-2 and DNA ploidy

	Aneuploidy		Aneuploidy
EGFR (+)	12/18 (66.7%)	c-erbB2 (+)	12/19 (63.2%)
EGFR (-)	20/36 (55.6%)	c-erbB2 (-)	20/35 (57.1%)

4. Expression of EGFR and c-erbB-2, nuclear DNA content and lymph node metastasis (Table 4)

Analysis of the relation between the expression of EGFR or c-erbB-2 and nuclear DNA content in the primary cancer focus revealed positive lymph node metastasis in 19 (86.4%) of 22 cases with the expression of EGFR in the primary focus. In contrast, of 40 cases without the expression of EGFR, only 19 (43.2%) had positive lymph node metastasis. Thus, the frequency of positive lymph node metastasis was significantly higher ($p < 0.01$) in cases with the expression of EGFR in the primary focus. The cases with the expression of c-erbB-2 and the cases with DNA aneuploidy both showed a higher tendency of lymph node metastasis. Therefore, the expression of EGFR in the primaty site was classified by the depth of invasion and the relation with lymph node metastasis was analyzed (Table 5). The frequency of lymph node metastasis in cases of early cancer with positive EGFR expression of was 3/5 (60%), which was significantly higher ($p < 0.05$) than that of 1/16 (6.3%) in the group of cases with negative EGFR expression. There was a similar tendency in advanced cancers but no significant difference was found. Fig. 1 shows a case of an early sm gastric cancer, which was EGFR-positive and revealed positive lymph node metastasis reaching N2.

Table 4. Expression of EGFR and c-erbB-2, nuclear DNA content and lymph node metastsis

	n (+)		n (+)
EGFR (+)	19/22 (86.4%) *	c-erbB2 (+)	13/20 (65.0%)
EGFR (-)	19/40 (43.2%) *	c-erbB2 (-)	25/42 (60.0%)

* $p < 0.01$

	n (+)
Aneuploidy	21/32 (65.6%)
Diploidy	12/22 (54.5%)

Table 5. Correlation between lymph node metastasis and EGFR expression according to death of invasion

1) m•SM (n = 21)		2) pm- (n = 41)	
	n (+)		n (+)
EGFR (+)	3/ 5 (60.0%)	EGFR (+)	16/17 (94.1%)
EGFR (-)	1/16 (6.3%)	EGFR (-)	18/24 (75.0%)

* $p < 0.05$

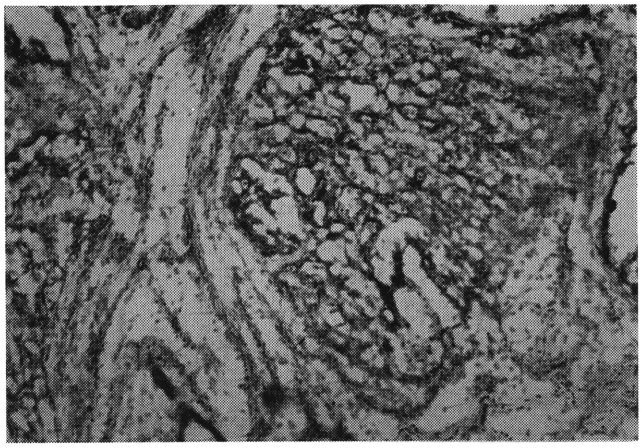

Fig. 1. A case of early gastric carcinoma, which was EGFR positive and had lymph node metastasis reaching N2

5. The expression of EGFR and c-erbB-2 in the primary and lymph node metastatic sites (Table 6)

The expression of EGFR and c-erbB-2 in the primary and lymph node metastatic sites was studied in 17 cases. Of 11 cases with EGFR expression in the primary site, 9 had the expression of EGFR in the metastatic site of the lymph node as well, However, out of 6 cases without EGFR in the primary site, 4 had the expression of EGFR in the lymph node metastatic site. Regarding c-erbB-2, all the 6 cases with the expression of c-erbB-2 in the primary site had the expression in the lymph node metastatic site. Of 11 cases without expression of c-erbB-2 in the primary site, 4 had the expression in the nodal metastasis. For the both of EGFR and c-erbB-2, the frequency of positive expression was higher in the lymph node metastatic site than in the primary site.

Table 6. Expression of EGFR and c-erbB-2 in the primary and lymph node metastatic sites

1) EGFR

primary	metastasis	
+	+	9/11 (81.8%)
+	-	2/11 (18.2%)
-	-	2/ 6 (33.3%)
-	+	4/ 6 (66.7%)
11/17 (64.7%)	13/17 (76.5%)	

2) c-erbB2

primary	metastasis	
+	+	6/ 6 (100%)
+	-	0
-	-	7/11 (63.7%)
-	+	4/11 (36.4%)
6/17 (35.3%)	10/17 (58.8%)	

6. Growth cycle and the intensisty of expression of EGFR

The relative intensity of expression of EGFR in the stationary and logarithmic growth phases of MKN-28 was shown in Fig. 2. As compared with the logarithmic growth phase, the intensity of the expression of EGFR was lower in the stationary phase.

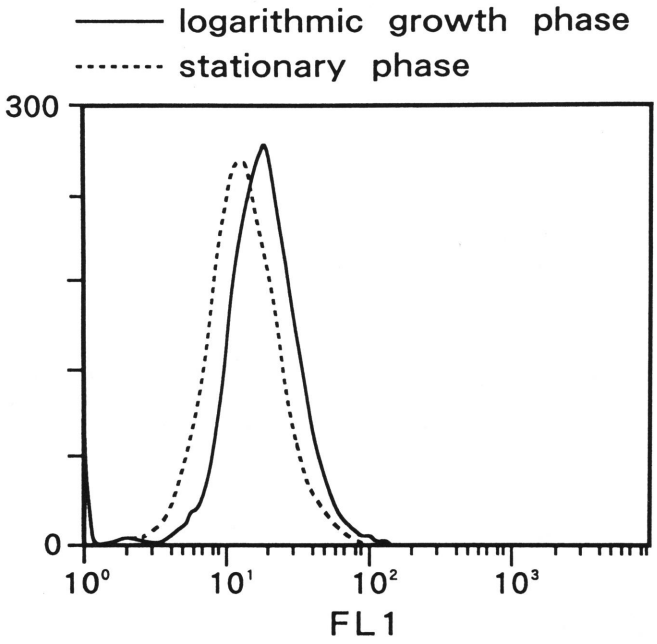

Fig. 2. Correlation between growth cycle and the intensity of expression of EGFR

7. Change in the expression of EGFR in an MKN-28-lymphocyte mixed culture system

When MKN-28 and PBL were incubated together at a ratio of 1:6, the growth of MKN-28 was suppressed as compared with the control group as shown in Fig. 3. The intensity of the expression of EGFR was rather greater than in the mixed culture system. In the comparison with the group in which PBL and PHA were added (Fig. 4), the tumor cell growth was more markedly inhibited than in the PBL

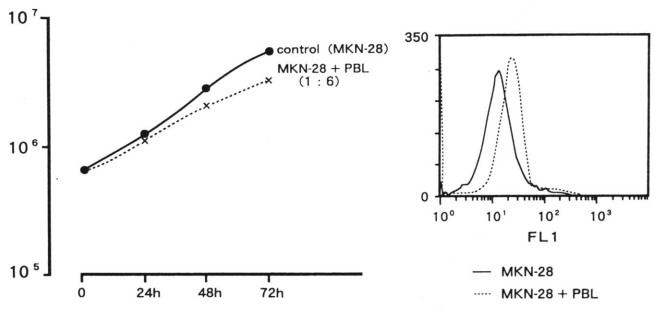

Fig. 3. Changes in the expression of EGFR of MKN-28 by mixed culture of PBL

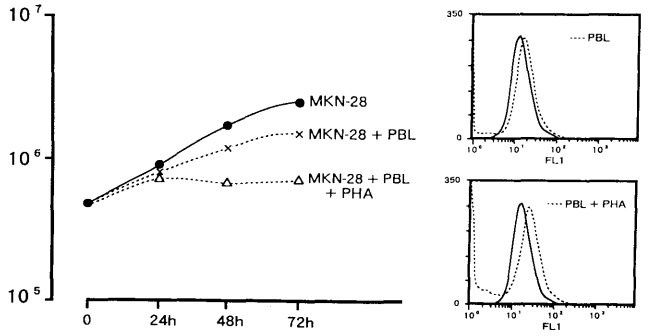

Fig. 4. When PHA was added, the tumor cell growth was more markedly inhibited than in the PBL mixed culture group and the intensity of EGFR expression also more increased.

mixed culture group and the intensity of the expression of EGFR also showed a further increase. Similarly, when MKN-28 was incubated with lymphocytes from the metastasis-negative lymph node of a patient with stomach cancer, the growth of MKN-28 was inhibited and the expression of EGFR was also facilitated. Such changes were found in 2 of 6 cases. In the other 4 cases, neither the growth curve nor the intensity of expression of EGFR was affected.

Discussion

Epidermal growth factor (EGF) is a growth factor consisting of 53 amino acids residues as identified from the submaxillary glands of male by Cohen in 1962.[5] Thisgrowth factor promotes the proliferation and differentiation of epithelial cells and mesenchymal cells. Epidermal growth factor receptor (EGFR) is a receptor for EGF and TGF α , being a glycoprotein with a molecular weight of 170,000. It consists of an extracellular ligand-binding domain, a membrane-permeating domain and an intracellular domain having a tyrosin-specific protein kinase, and is thus structurally analogous to c-erbB protein.[7] The over-expression of EGFR was first recognized by Fabricant in 1977 in A431 cells which are squamous cell carcinoma cells derived from the human labia pudendi.[8] Thereafter, the expression of this receptor was found not only in various squamous cell carcinomas such as carcinomas of the esophagus, [9]uterine cervix,[10] etc. but also in brain tumors,[11] inclusive of glioma, stomach cancer,[12] breast cancer[1] and colon cancer,[12] and the correlation with biological malignancy has for some time been a focus of attention. On the other hand, c-erbB-2 protein is a glycoprotein which is structually analogous to EGFR and has been suspected to be a membrane receptor[13] but its ligand has not been indentified. The over-expression of c-erbB-2 has been reported in patients with breast cancer[3,4] and stomach cancer. As far as breast cancer is concerned, there are many reports suggesting the relationship between nodal involvement and the prognosis. Kokai et al.[5] demonstrated in vitro that c-erbB-2 and EGFR cooperate to transform a fibroblast cell line, suggesting a possible interaction of these two protooncogenes. Therefore, the expression of EGFR and c-erbB-2 proteins in stomach cancer tissues was evaluated in this study. The expression of EGFR showed a higher incidence in advanced cancer than in early cancer but no disparity was found between histologic types. Moreover, the expression of EGFR was well correlated with nodal involvement, and in particular in the group of early cancer. And also the node metastasis in cases with the positive EGFR expression in the primary site was significantly seen in a higher incidence than that in cases without the expression. When the expression of EGFR was compared between the primary sites and the metastatic nodes, the expression in the metastatic site was apparently higher than that in the primary one and 4 out of 6 without the expression of EGFR in the primary site showed the positive expression in the metastatic site. Two possible reasons may be proposed. One is that the expression of EGFR in the primary site is mosaic so that only the cells with the expression of EGFR are selectively involved at metastasis. The other is that cells without the expression of EGFR begin to express EGFR in the process of metastasis.

With regards to c-erbB-2 protooncogen, the expression was rather significant in early cancer according to the depth of cancer. By histological types, the expression rate was higher in well differentiated carcinoma than in undifferentiated one. Regarding the relationship with nodal involvement, the node metastasis was more frequently encountered in cases with positive c-erbB-2 expression in the primary site and positive c-erbB-2 expression was more frequently seen in the metastatic site than in the primaty site. Thus, as seen in the expression of EGFR, it was suspected that there should be a close relationship with lymph node metastasis. It was assumed that positive c-erb-B-2 expression imply a presence of nodal involvement not only in the progression stage of carcinoma but also in the initiation stage.

Since Hedley et al.[15] reported the method for analysis of nuclear DNA from paraffin-embedded tissues in 1983, the analytical method of nuclear DNA content has been clinically used widely for predicting biological malignancy. In the present investigation, the frequency of DNA aneuploidy was high in cases of advanced cancer, differentiated cancer and lymph node metastasis, but there was no correlation with the expression of EGFR or c-erbB-2 protooncogenes. These results suggested that the expression of EGFR and c-erbB-2 should be an independent factor of DNA ploidy and particularly the expression of EGFR seemed highly correlate with lymph node metastasis. Establishment of lymph node metastasis is the base on the liberation of cancer cells from the primary focus, invasion into the blood vessels, migration, and settlement and proliferation in the lymph node. Therefore, in order to clarify the role of EGFR protooncogene in the mechanisms of metastasis, a co-

incubation study using MKN-28 cells and cells in mixed culture with lymphocytes was inhibited accompanying a relative increase in the expression of EGFR in MKN-28. Regarding the relation between cell cycle and expression of EGFR, the intensity of EGFR expression in MKN-28 was more reduced in the stationary phase than in the logarithmic growth phase. On the basis of the above fact, it is contemplated the cells whose growth was suppressed by mixed culture with lymphocytes should have shown a decreased EGFR expression. In contrast, the result in this series clarified that there was a tendency toward an intensified EGFR expression of cells in the culture with lymphocytes. There are two possibilities. One is that generation of EGFR protoonegene in MKN-28 was increased by the direct action of lymphocytes or the interaction of cytokines. The other possibility is that MKN-28 cells with weak EGFR expression were more susceptible to growth inhibition by lymphocytes, meanwhile cells with EGFR expression are resistant to growth inhibition. On the basis of tha fact that the expression of EGFR was apparently enhanced as a result of proliferation of cells with intensified EGFR expression. As to the latter possibility, attention may be directed to the report that NIH 3T3 cells with amplified c-erbB-2 oncogene show resistance to the cytotoxic effect of TNF α and macrophages.[16] It may, thus, be postulated that EGFR, too, is similarly resistant to lymphocytes and cytokines.

Development of nodal involvement is explained that cancer cells liberated from the affected gastric tissue migrate into the lymph vessels, settle and proliferate in the lymph node. As a result, the cells with the expression of EGFR act as an antagonist of lymphocytes, thus facilitating the occurrence of metastasis. The immunostaining study clarified the fact that EGFR is in close association with involved lymph nodes in stomach cancer.

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