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Effects of Gastric Cancer Cells on Lymphocyte Proliferation

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SUMMARY **Lack of lymphocyte infiltration into gastric cancer tissue appears to be an ominous prognostic indicator. The effects of gastric cancer cells on PHA-induced lymphocyte proliferation were studied. Peripheral lymphocytes were co-cultured for 72 hours with either gastric cancer cells or normal mucosal cells. Pairs of cancerous and normal mucosal cells from stomachs of eight patients, were separately co-cultured with peripheral lymphocytes either from patients or from normal volunteers. The degree of PHA-induced lymphocyte proliferation was measured by ^{3}H-thymidine incorporation. The lymphocyte proliferation was inhibited by the presence of either gastric cancerous or normal mucosal cells in a dose-related manner. The lymphocytes from the normals proliferated twice as much as did the lymphocytes from the patients. The isotope incorporation occurred in lymphocytes rather than in gastric cells since the later incorporated insignificant amounts of isotope. There was no difference between gastric cancerous or normal mucosal cells inhibiting the proliferation of either normal or patients' lymphocytes ($p > 0.05$). In conclusion, gastric cancerous cells (up to 10^{6}/ ml) have no enhanced inhibition on lymphocyte proliferation when compared with normal gastric mucosal cells.**

The prognostic significance of the lymphocyte infiltration in a tumor has attracted many pathologists' attention. In 1912, MacCarty *et al.* [1] first reported the favorable prognostic value of lymphocyte infiltration in gastric cancer, which probably reflected the consequence of an ongoing immune response against malignant cells. This observation was later confirmed by several authors. [2-4]

Some considered that the gastric cancers with lymphocyte infiltration belonged to a distinct pathologic entity which may be separated from the common type of gastric cancer. [4] Its incidence is around 1.3-4% in all gastric cancers. [5] It is possible that common type cancer cells, which lack lymphocyte infiltration, may produce certain factors capable of inhibiting lymphocytes. This possibility was herein studied by incubating lymphocytes with either cancer cells or normal gastric mucosal cells. In this study we evaluated the effects of gastric cancer cells on lymphocyte proliferation by co-culture of gastric cancer or non-cancer mucosal cells with peripheral lymphocyte either from cancer patients or from normal subjects.

MATERIALS AND METHODS

Patients

This study included eight patients with histologically confirmed adenocarcinoma of the stomach undergoing total or subtotal gastrectomy in the Department of Surgery, Veterans General Hospital, Taipei, Taiwan (Table 1). They received neither chemotherapy nor radiotherapy prior to the operation. They also received no medication within two weeks before the operation.

Lymphocytes

Heparinized peripheral blood was obtained from eight gastric

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Table 1. The clinical status of eight gastric cancer patients

Case No.	Sex	Age	Tumor location	Postsurgical stage	
1	F	59	Mid–body	Stage III ($pT_3N_1M_0$)	P
2	M	70	Antrum	Stage III ($pT_3N_1M_0$)	P
3	M	56	Antrum	Stage I ($pT_1N_0M_0$)	M
4	M	56	Antrum	Stage III ($pT_2N_2M_0$)	W
5	F	47	Antrum	Stage III ($pT_3N_2M_0$)	P
6	M	86	Antrum	Stage II ($pT_3N_0M_0$)	W
7	M	73	Mid–body	Stage III ($pT_3N_1M_0$)	P
8	M	63	Mid–body	Stage III ($pT_2N_1M_0$)	W

P : Poorly differentiated, M : Moderately differentiated,
W : Well differentiated.

cancer patients and eight healthy donors. These patients were not the same subjects as the tissue donors. The lymphocytes were separated by Ficoll-Hypaque density gradient centrifugation as previously described.[6] The cells at 1×10^6/ml were suspended in RPMI-1640 containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cell population thus obtained consisted of approximately 85-90% lymphocytes, 10-15% monocytes and less than 5% neutrophils. The viability of cells obtained was more than 95% by trypan blue exclusion.

Tumor cells

Gastric specimens were taken from cancer tissues as well as from normal gastric mucosal tissues which were 6 cm apart from the cancer tissue. Histopathologic confirmation was made retrospectively. Immediately following resection, the specimens were rinsed with normal saline and quickly sent to the laboratory for study.

Mucosal layers of the specimen were separated and were cut into small pieces in Hanks' balanced salt solution (HBSS). They were incubated with 100 μg/ml of trypsin (Sigma Chemical Co., St. Louis. MO, USA) at 37° C for 60 minutes. The supernatants containing many red blood cells were discarded and the fragments were further incubated with the same concentration of trypsin. The supernatants containing separated mucosal cells were collected every 15 minutes, and were replaced by the solution containing trypsin. The pooled cells were washed twice with HBSS.

Only cell preparations with more than 80% viability (by trypan blue exclusion) and with less than 5% obvious contamination from non-malignant cells, were used for experiments. Gastric non-cancer mucosal cells were prepared in the same way.

Co-culture with lymphocytes and gastric cells

Lymphocytes 1×10^5/well were cultured with gastric cells (1×10^3 to 1×10^6/ml for 3 to 6 days in the presence of 1 μg/ml PHA (Burrough Wellcome, Beckenham, UK) in flat-bottomed tissue culture plates (#3072; Falcon, Becton Dickson & Co. Osnard, CA, USA). The gastric cells were pretreated with mitomycin C (50 μg/ml) at 37° C for 30 min. The cells were incubated for 3 days in a humidified 5% CO_2/air incubator.

In the terminal 4 hours of culture, the cell mixture was pulsed with 0.5 μCi/well of ^{3}H-thymidine (6.7 Ci/mmol, New England Nuclear, Boston, MA, USA). The incorporated isotope was harvested in a semiautomatic cell harvester (model M-24S, Brandel, Gaithersburg, MD, USA). The incorporated isotope was measured by a standard toluene-based scintillation counting technique.[7]

Statistical analysis

All determinations in cell cultures were made in quadruplicate. The results were calculated as the mean ± SD. Paired comparison was made by Wilcoxon signed rank test.

RESULTS

Effects of PHA on cell proliferation

A previous report showed that 1 μg/ml of PHA is the optimal concentration for lymphocyte proliferation.[6] PHA-stimulated lymphocytes from gastric cancer patients had 50% less proliferation than lymphocytes from normal volunteers (n = 8) either co-cultured with gastric normal mucosal cells or with cancer cells (Figure 1). Either gastric cancer cells alone or normal gastric mucosal cells alone, in the presence of PHA, incorporated insignificant amount of ^{3}H-thymidine (Table 2).

Inhibition of lymphocyte proliferation

The lymphocyte proliferation was inhibited by the presence of gastric cancer or normal mucosal cells in a dose-related manner (Figure 1). When incubated with normal lymphocytes, there was no difference between cancer cells and normal mucosal cells in inhibiting lymphocyte proliferation ($p > 0.05$). The same phenomenon was observed when a patient's lymphocytes were used.

DISCUSSION

The effects of gastric cancer cells on immunity were studied. As shown in Figure 1, the presence of gastric cells in lymphocyte cultures inhibited the PHA-induced ^{3}H-thymidine incorporation. The

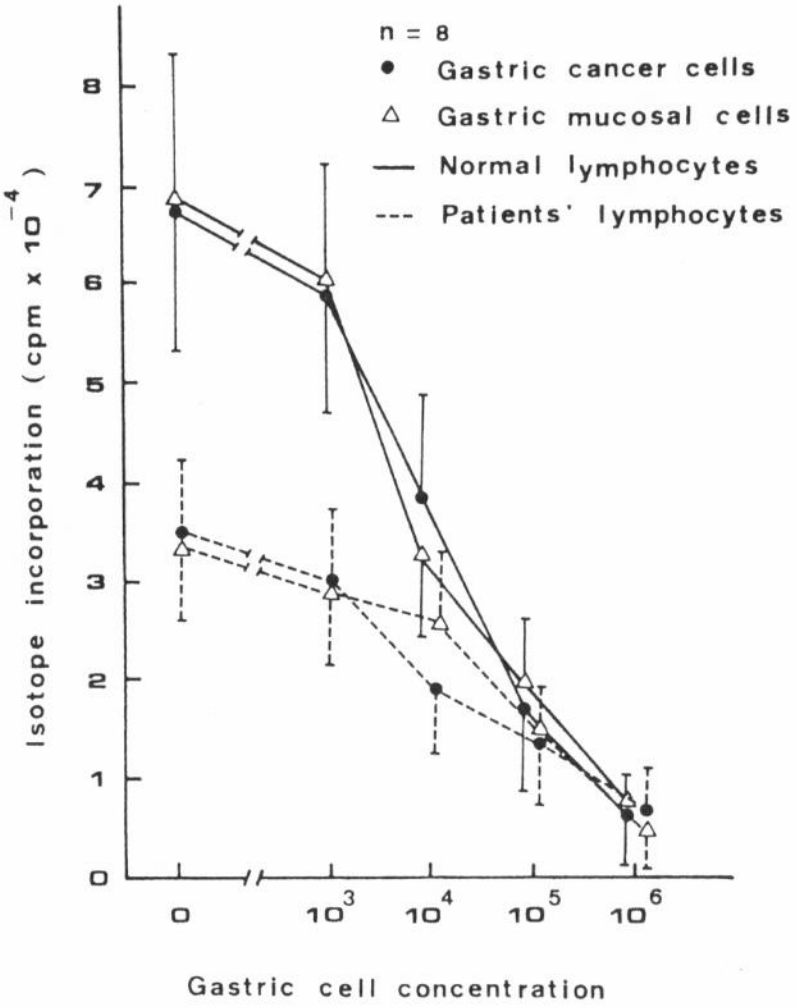

Fig. 1 Co–cultures of mitomycin–C treated gastric cells with normal lymphocytes and patients lymphocytes (1×10^5 cells/ml).

Table 2. The effect (cpm) of PHA stimulation on gastric cancer or non–cancer cells.

Cell type	PHA (1 μg/ml)	Cell concentration 10^3	10^4	10^5	10^6
Cancer cells (n=6)	+	137.3	159.5	351.5	652.0
	–	160.7	229.3	329.7	508.3
Non–cancer cells (n=6)	+	158.3	202.0	374.2	422.0
	–	167.6	187.6	373.4	423.2

inhibition was related to the concentration of gastric cells. Both cancer cells and normal mucosal cells inhibited the isotope incorporation to a similar degree.

In the total isotope incorporation by the co-cultured cells, the amount of isotope incorporated by gastric cells was negligible. As shown in Table 2, the isotope incorporation by gastric cells was of minute quantity. The increase in incorporation was related to cell number. As shown in Figure 1, the isotope incorporation in the lymphocyte co-cultures decreased rapidly when the gastric cells increased. These results indicate the decrease in isotope incorporation in the co-cultures is due to the inhibition of lymphocytes by the presence of gastric cells.

As reported by Orita *et al.*[8], it is worthwhile of mention that lymphocytes from gastric cancer patients were poorly stimulated by PHA when compared with normal lymphocytes in the same culture condition (Figure 1). There are two possibilities for this. First, lymphocytes from patients were inhibited by certain humoral factors from cancer tissues. One of the most potent natural immune-inhibitors is arginase.[9] Second, patients' lymphocytes themselves were defective in some way.

It is likely that the arginase released from gastric cells inhibited the lymphocyte proliferation. However, it was shown in our laboratory that gastric cancer tissues contain more arginase than normal gastric mucosal tissues (unpublished data). These results conflict with the results of Figure 1. As shown in Figure 1, cancer cells and normal gastric mucosal cells inhibit lymphocyte proliferation to a similar degree. Based on these observations, arginase probably plays an insignificant role in inhibiting lymphocyte proliferation. This needs further confirmation. The involvement of other humoral factors currently cannot be excluded.

It was our purpose to investigate the possible inhibitory role of cancer cells in the immunity. However, in the co-culture study, the cancer cells did not show more inhibitory effect on lymphocyte proliferation than normal gastric cells. The problem of whether or not the cancer cells exert more inhibition on immunity is still not settled. The failure to show enhanced cancer cell inhibition on lymphocytes could be explained in several ways. (1) The mitogen (PHA, 1 μg/ml) used in this study was too strong to show the differential inhibition caused by gastric cells. (2) In a natural *in vivo* condition, the lymphocytes infiltrating into the cancer tissue would be surrounded by numerous cancerous cells.[2-4] In this co-culture study, the number of cancer cells was not great enough to cause a substantial degree of inhibition. Hence the difference between normal mucosal cells and cancerous cells was not evident. It is almost impossible to further increase the gastric cell number since the addition of a higher number of gastric cells may cause a marked degree of lymphocyte inhibition and hence again the differential effect of normal and cancer cells can not be seen. To attack this problem, a study with cell extracts may provide meaningful information. This study is currently under way.

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