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HLA Class I and Class II Antigen Expression in Gastric Cancer Cells and Induction of Its Expression by Local Injection of OK-432

Daikichi Okada

First Department of Surgery, Nagasaki University School of Medicine

The measurements of HLA class I and II antigen expressions were made in association with assessment of antiproliferation effects on cancer cells.

Experiments were designed to measure HLA class I and II expressions by using flow cytometry in the mixed culture of WiDr cells, OK-432 and peripheral lymphocytes.

The result showed that proliferation of cancer cells was suppressed in contact with OK-432, indicating augmented HLA class I and II antigen expression. It was suggested of activation of immunocompetent cells as well as induction of HLA antigen expression.

HLA class I and II antigen expressions were clinically eveluated in patients with gastric cancer between with and with-out local administration of OK-432. Cancer tissues were fixed by to AMeX method and immunohistochemically stained by ABC method. The degrees of HLA class I antigen expressions were depressed in accordance with cancer progression. In contrast, they were seen in the majority of patients in this study. On the other hand, HLA class II antigen expressions were reduced ($p < 0.05$) with cancer progression, the increase in cancer infiltration and the decrease in differentiation.

It was suggested that reduction of HLA-class II (DR) antigen expression be in association with biologically malignant potential.

In addition, it was assumed that local administration of OK-432 enabled HLA class II antigen expression and lymphocyte infiltration around the tumor to enhance even in patients with advanced and undifferentiated cancers and it was suggested that OK-432 made it possible to induce tumor immunity and to become a valid method of postoperative immunotherapy.

Introduction

It is known that the activation of CTL and helper T in tumor-bearing host necessitates MHC and HLA antigen expression, recognizing tumor-specific antigens.[1,2] The possibility exists that proliferation of cancer cells and promotion of metastasis are in association with suppression of HLA antigen expression.[3-11]

In this experimental study, it was aimed at elucidating a correlation with interaction of host immunity and proliferative cancer cells.

In this series, the cytocidal effect of immunocompetent cells was experimentally evaluated in relation to HLA class I and II antigen expressions. In cotrast, HLA class I and II antigen expression was immunohistochemically compared with hostopathologic factors. The effects of OK-432 on HLA class I and class II antigen expressions were assessed in comparison with Tumor-infiltrating lymphocytes (TIL).

Material and Method

Experiment 1:

HLA antigen expression in cultured cells and response to immunocompetent cells.

WiDr cells originated from colon cancer cells were used as a target cell and peripheral blood lymphocytes (PBL) as an effector cell. And BRM of OK-432, rHu IFN, anti-IFNγ Ab was used. MoAb of W6/32 (anti-HLA-class I) and of Tal 1B5 (anti-HLA-class II) were selected.

Method (1) WiDr cells were cultured in MEM, divided into 4 groups by the addition of PBL (E:T = 2:1), OK-432 0.1KE/ml and rHuIFN 10u/ml and mixcultured for 48 to 96 hours at 37℃ in 5% CO_2. Cells were counted, added mouse serum to interfere with non-specific reaction, contacted with MoAb, followed by FITC conjugated anti-mouse IgG Ab. In each step, the sample was rinsed with PBS, and centrifuged twice. The amount of fluorescence of HLA antigen expression on the surface of cells was measured by using flow cytometry (FACS IV).

(2) 1×10^6 WiDr cells were taken and effector cells were prepared by excluding macrophages with adherence method. The samples were divided into three groups, control (E:T = 2:1), OK-432 0.1KE/ml (E:T = 2:1), and OK-432 0.1KE/ml (exclusion of Mϕ), cultured for 72 to 96 hours and the fluorescence amount was measured by flow cytometry according to indirect detection of fluorescent antibody method.[4]

(3) 1×10^6 WiDr cells were divided into three groups:

control, in contact with OK-432 0.1KE/ml and OK-432 0.1KE/ml adding anti-INFγ antibody, respectively. The samples were cultured for 72 to 96 hours. And also flow cytometry was used for the measurement of fluorescence.

(4) The IFNγ titers were measured in a mixed culture of WiDr cells with OK-432, PBL and IFNγ.

Experiment 2:

HLA antigen expression in resected gastric cancer tissues.

1. Subject (Table 1)

The gastric cancer tissues resected at the First Dept. of Surgery, Nagasaki University Hospital and its affiliated hospitals were subjected to this study in which 15 were OK-432 injected cases and the other 33 belonged to not injected. OK-432 was endoscopically given around the tumorous lesion 3 to 7 days prior to operation.

Table 1. Clinical background

	I local administration of OK -432 15 cases	II control 33 cases
age	38 to 81 (mean 61.2)	28 to 83 (63.7)
sex	M/F = 9/6	M/F = 22/11
histology		
tub_1	5	5
tub_2	1	13
por	7	15
sig	2	
stage		
I	7	9
II	4	5
III	1	12
IV	3	7

2. Method (Table 2)

Immediately after the resection of the tumor-bearing stomach, tissues were paraffin-embeded. A part of slice was stained with HE and the others were immunohistochemically stained according to Avidine-Biotin-complex method[16] with the use of MoAb (anti-HLA-ABC, W6/32, anti-HLA-DR, TAL 1B5, DAKO JAPAN.)

3. Estimation

The stainings were regarded as (+) 25 to 50% staining, (++) 50 to 75% (+++) over 75%. When compared with the positive control of Mϕ and the endothelium under light microseopic finding. TIL were graded as (++) definitively rich infiltrative cells, (+) similar degree, (±) scanty as compared with those in non-cancer tissues.

Table 2. Experimental protocol

I	OK-432 local administration endoscopically given 0.4cc diluted 2 ~ 3ml of 5KE OK-432 in 5 sites of intra-and around the tumors.
II	Sampling and treatment
	① Slices of 2 ~ 3mm
	② 4℃ in aceton for 30min
	③ Overnight in -20℃ aceton
	④ 4℃ in aceton for 15min
	⑤ Kept at room temperature in aceton
	⑥ Kept at room temperature in Methyl benzoate
	⑦ Kept at room temperature in Xylene
	⑧ paraffin enbedded
III	histochemical staining
	① 5μ slice
	② deparaffinized with Xylen, Acetone
	③ intrinsic peroxidase block
	④ NSS at room temperature
	⑤ contact with MOAbs at 4℃ overnight
	⑥ Second Ab (Biontinized) at room temperature
	⑦ ABC reaction at room temperature for 40min
	⑧ Paraffin enbedded preparate
ABC reaction	
DAB	
nuclear staining (hematoxyline)	
dehydration, inclusion	

Results

Experiment 1:

Proliferation of WiDr cells was suppressed in the mixed culture in contact with 0.1KE/ml OK-432 and also HLA-DR antigen expression was enhanced in proportion to the concentration of PBL as shown in Fig. 1.

In a mixed culture of lymphocytes and OK-432 after removal of Mϕ, suppression of proliferation of WiDr cells was weakened accompanying less HLA-A•B•C•DR antigen expression as shown in Fig. 2 as compared with that of unpretreated PBL alone. On the other hand, in a mixed culture with OK-432 and PBL in contact with anti-IFNγ. The proliferative curve of WiDr cells was almost the same as the control without enhancement of HLA antigen expression as shown in Fig. 3.

The IFNγ titers in supernatant of culture medium were affected by culture medium, time duration of culture and PBL. In cotrast, the concentration of IFNγ in the control remained stationary and it was less than 0.02u/ml in contact with PBL. And also no significant change in the concentration of IFNγ was seen regardless of the PBL concentration and the time duration of contact. In contact with OK-432 0.1KE/ml + PBL (E:T = 1:1), the IFNγ concentration showed 0.44 to 1.70u/ml and concentration with a 96 hour contact. In case of contact with OK-432 0.1KE/ml + PBL (E:T = 2:1), the IFNγ concentration was increased to 1.9 to 7.9u/ml, indicating a close correlation with the

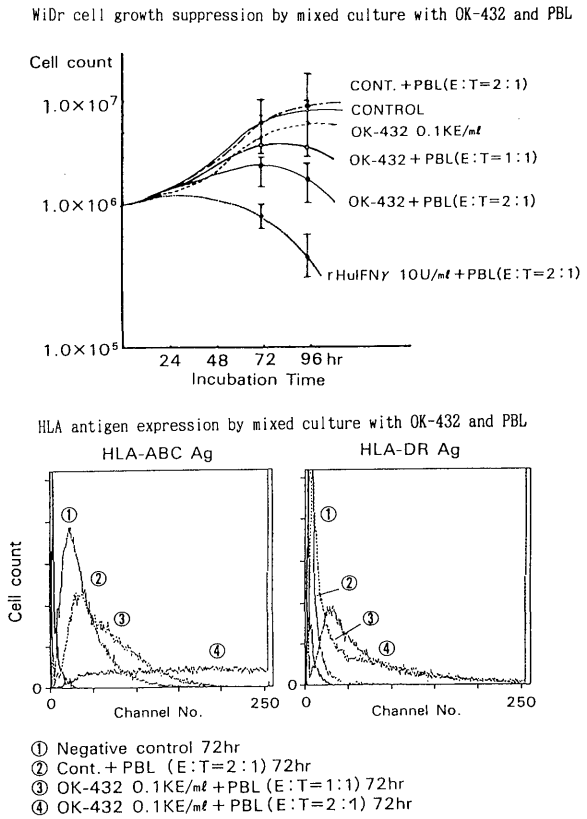

Fig. 1. Cell proliferation and changes in HLA antigen by mixed culter with OK-432 and lymphocytes

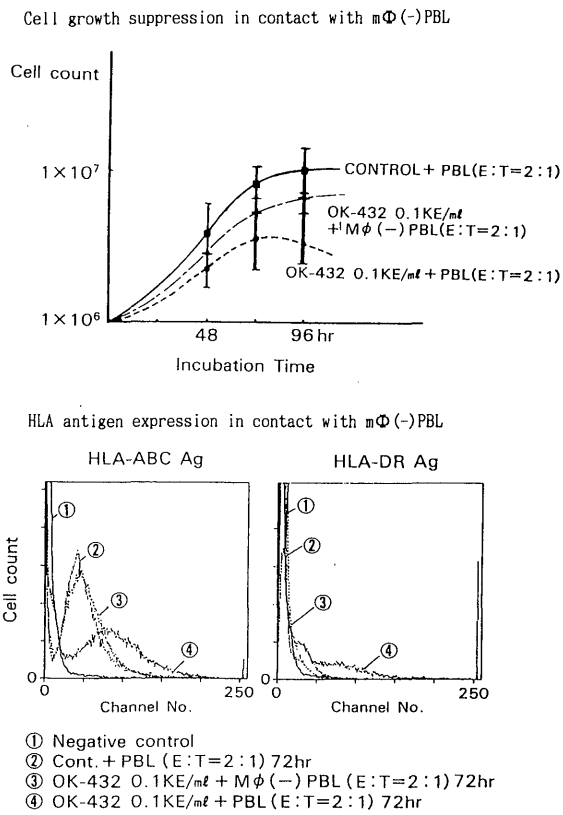

Fig. 2. Changes in cell proliferation and HLA antigen expression by lymphocytes exclusive of mΦ

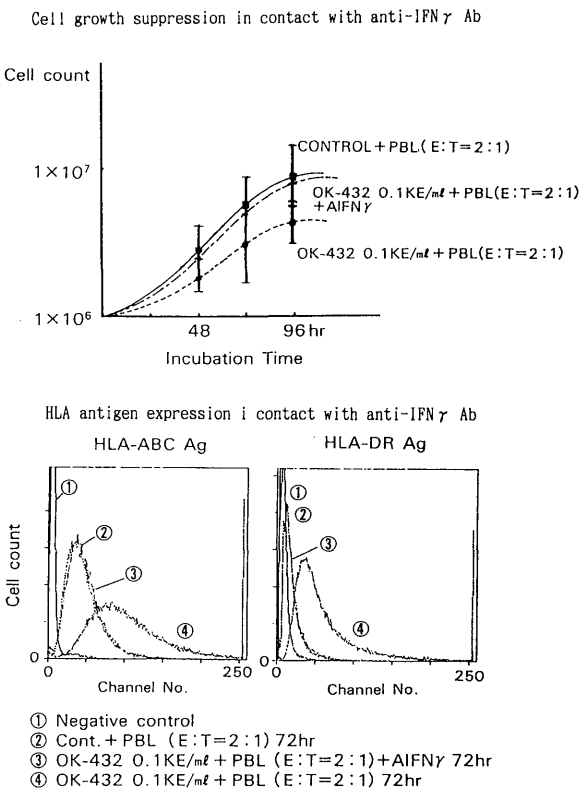

Fig. 3. Changes in cell growth and HLA antigen expression in contact with anti-INFγ Ab

concentration of PBL. Meanwhile, in case of contact with IFNγ 10u/ml and PBL, the IFNγ concentrations were significantly high, ranging 3.0 to 16u/ml, maintaining a high level as compared with those in the other groups. On the other hand, the IFNγ levels were similar to the control (0.06 to 0.16/ml) contact with PBL after removal of Mφ as shown in Table 3.

Table 3. IFN γ concentration in culter medium

the control	~0.02U/ml
OK-432 0.1KE + PBL (E:T=1:1)	0.44 ~ 1.70U/ml
OK-432 0.1KE + PBL (E:T=2:1)	1.9 ~ 7.9U/ml
INF γ 10U/ml + PBL (E:T/1:1)	3.0 ~ 16U/ml
OK-432 0.1KE + M Φ (-) PBL	0.06 ~ 0.16U/ml

Experiment 2:

HLA A•B•C and DR antigen expression of gastric cancer tissues.

(1) HLA antigen expression in relation to disease stages. (Fig. 4).

The intensity of HLA-A•B•C antigen expressions were gradually reduced with progression of disease stages. In contrast, HLA-DR antigen expression was seen only in early gastric cancer of stage I and II.

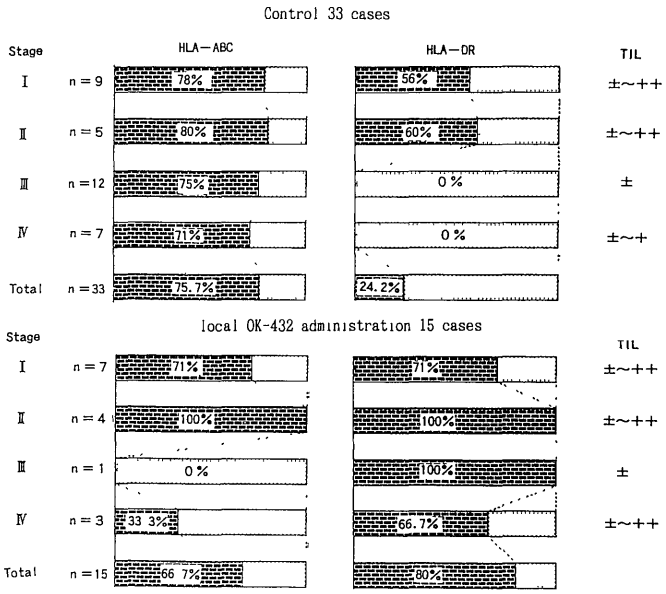

Fig. 4. Relationship between HLA antigen expression and stages, TIL

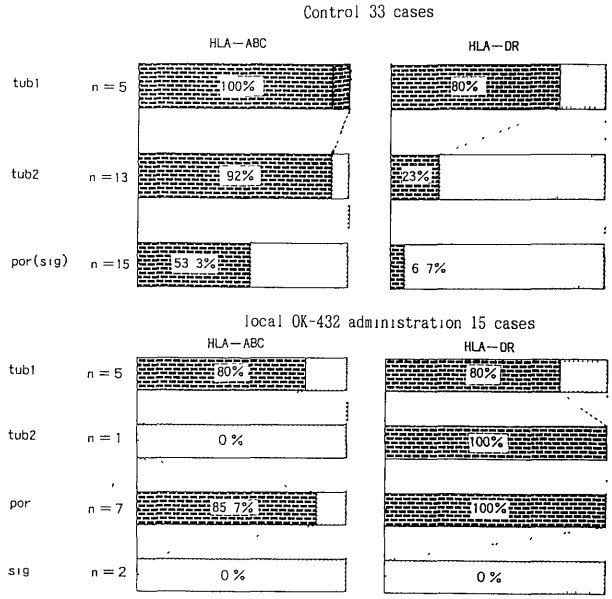

Fig. 5. Relationship between HLA-antigen expression and differentiation

(2) HLA antigen expression in relation to differentiation. (Fig. 5)

HLA A•B•C antigen expressions were seen in almost all of well differentiated carcinomas. Even in undifferentiated carcinomas, the HLA ABC antigen expressions were seen in 8 out of 15 (53.3%) in spite of a decrease in their expressions. HLA DR antigen expression was shown in 4 out of 5 (80%). In contrast, it was reduced in accordance with poor differentiation ($p < 0.01$) as shown in Fig. 5.

(3) HLA antigen expression in relation to the depth of cancer infiltration, invasive types and node metastasis. (Fig. 6, 7, 8)

There was no close correlation with HLA-A•B•C antigen expression and the depth, invasive types nodal involvement. On the other hand, a decrease in HLA DR antigen expression was seen in accordance with cancer progression. There was a significant difference ($p < 0.05$) in HLA DR expression between INF and n_2n_3. TIL was significant in some of stage I and II. However, there was no good correlation between TLL and HLA antigen expression.

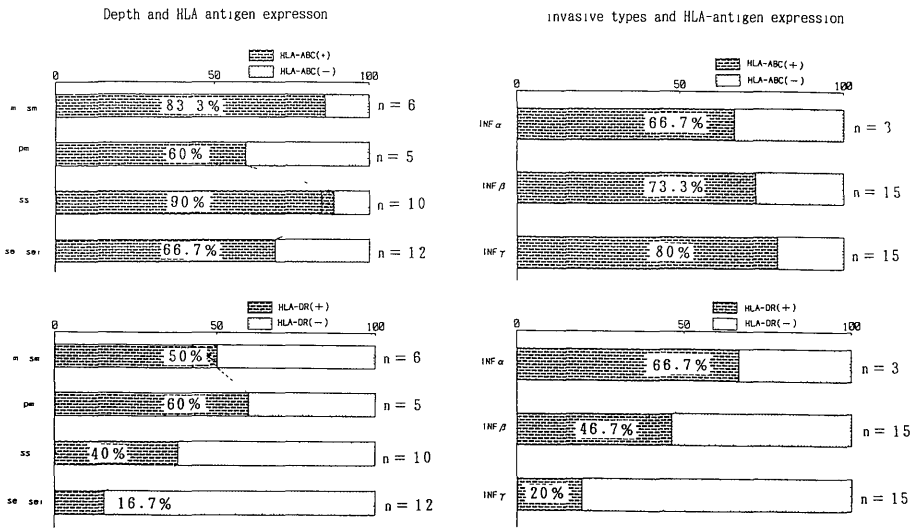

Fig. 6. Relationship between the depth invasive types and HLA-antigen expression

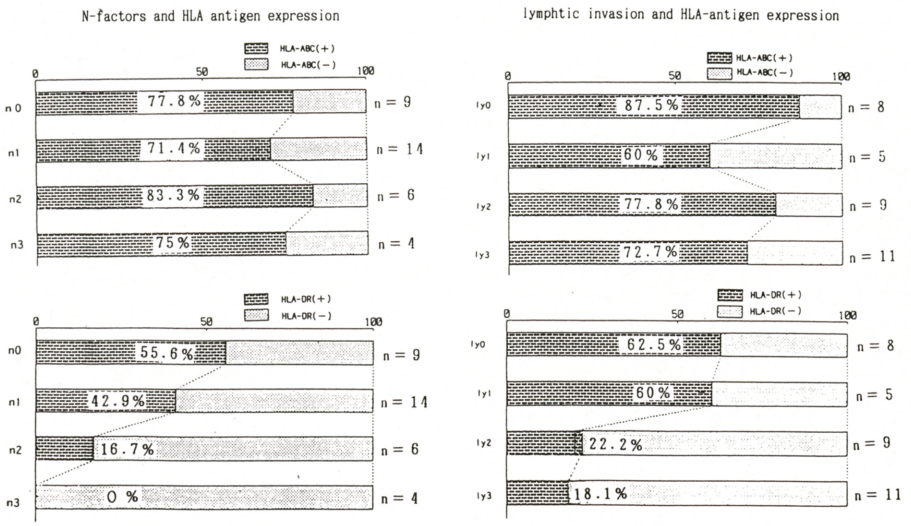

Fig. 7. Relationship between n-factors lymphatic invasion and HLA-antigen expression

Local administration of OK-432 and HLA-A•B•C-DR antigen expression

(1) Relation between disease stages and HLA-A•B•C antigen expression. (Fig. 4)
HLA-A•B•C antigen expression were high in 10 out of 15 (66.7%) in stage I and II. In contrast, these were reduced in one out of four (25%) in stage III and IV. On the other hand, HLA-DR antigen expression was seen in 12 out of 15 (80%) and it was seen in three out of 4 (75%) even in stage III and IV, showing there was no difference between stage I and II.

(2) Relation between differentiation and HLA antigen expression. (Fig. 5)
HLA-A•B•C as well as HLA-DR antigens were well expressed in well and poorly differenciated carcinomas.

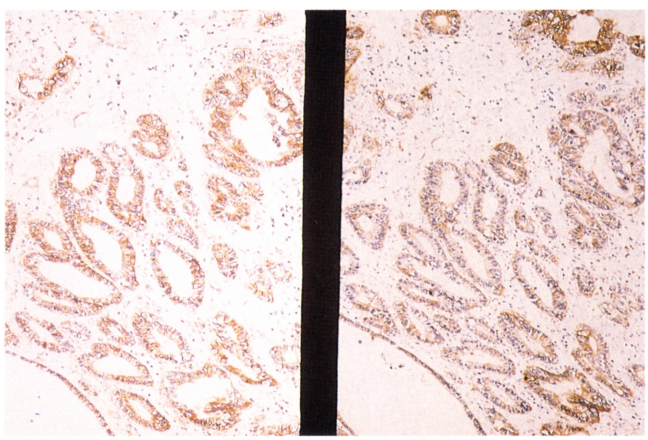

Fig. 2. Early gastric cancer (well differentiated) (left) HLA-ABC Ag, (right) HLA-DR Ag

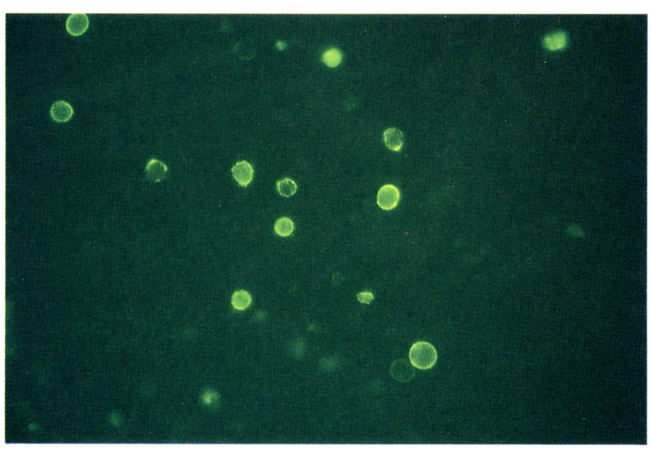

Fig. 1. OK-432 0.1KE/ml + PBL (E:T = 1:1) 72hr HLA-ABC Ag

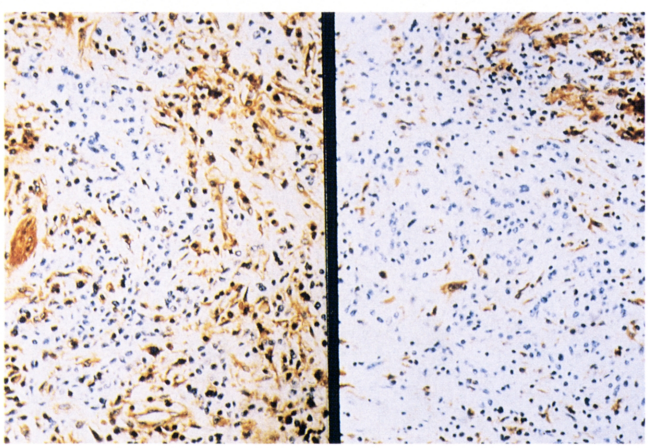

Fig. 3. Advanced gastric cancer (poorly differentiated) (left) HLA-ABC Ag, (right) HLA-DR Ag

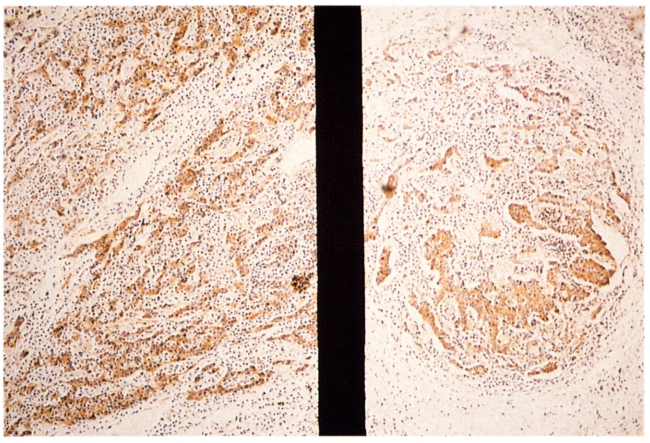

Fig. 4. Advanced gastric cancer poorly differentiated OK-432 locally given
(left) HLA-ABC Ag, (right) HLA-DR Ag

There were some of intensive expressions and cell infiltrations in poorly differentiated carcinomas on occasion. (Fig. 4) The grades of TIL had no close correlation with the grades of disease stage and differentiation, reflecting the fact that local injection of OK-432 produces a reflactory inflammation. These were enhanced in cases of positive expressions against HLA-A•B•C and DR antigen. As compared with the control, HLA-DR antigen expressions were induced and immunologic cell infiltrations also were enhanced in the cases with local OK-432 injection.

Discussion

It is accepted that immunotherapy is the most important tool to manage cancer patients with multimodality therapy. Recent studies[1)] clarify that MHC and HLA antigen expressions are essential for recognizing weak tumor-specific antigens and for having cytocidal effect of cytotoxic and helper T cells on cancer cells.

HLA antigens were divided into the two: class I and II antigens. Class I antigen exists in the nucleus and control cytotoxic T cell function[17)] and its expression is depressed and/or diminished with tumor progression of breast and renal cancers.[3-11)]

On the other hand, HLA class II (DR) antigen affects the function of helper T cells and it is expressed in the immunocompetent cells such as macrophages and endothelial cells, in contrast, hardly seen in the normal cells.[1, 18, 19)]

In this study, HLA antigen expressions were evaluated in terms of histopathologic factors and host immunodefensive mechanism of TIL and also induction of HLA antigen expression was assessed by endoscopically local OK-432 administration into gastric cancer tissues prior to operation.

HLA-antigen expressions on the cell surface were evaluated to elucidate the influence of OK-432 and PBL as an effector cells on cultured cells from the suppressive effect of proliferative cells.

It is well known that WiDr cells as a target cell used in this study have HLA-A•B•C antigen expression despite not having HLA-DR antigen expression which is enhanced by contact with IFNγ in proportion to its concentration and result in suppressive effect of cell proliferation. And also it is defind that OK-432 activates macrophages, NK activity and IFN production.[20)]

Although the cytocidal effect by OK-432 was not clear in this study, HLA-antigen expressions were enhanced and cell proliferation was suppressed in a mixed culture with target cells. The maximum was 0.1KE/ml of OK-432 in a 72 hour culture.[21, 22)] When exceeded the OK-432 concentration of 0.1KE/ml, these effects were in proportion to the rates of effector cells.

The mechanism of OK-432 cell-mediated effect is that OK-432 first activates macrophages and T cells are activated through its stimulation of IL-1, as a result, cytokines of IFNγ and IL-2 were generated and finaly induced and enhanced HLA-class I•II antigen expression.

In this study, there was no evidence that T cells directly attack target cells. However, it is suggested that Mϕ and INFγ plays more important role in activated cytocidal effect and suppressed cell proliferation rather than various cytokines.[23)] The roles of IFNγ which is generated by helper T cells are augmentation of HLA-class I antigen expression, induction of HLA-class II antigen expression and activation of immunocompetent cells with varying variety as compared with IFNα, β.[24, 25)]

The presence of INFγ is indispensable for the function of antitumor immunity. It is said that helper T cells fail to recognize tumor cells but are capable for recognition of them. In spite of this fact, CTL alone are unable to recognize tumor-associated antigen (TAA) and to represent cytocidal activity. At present, it is accepted that Mϕ first plays a key role in the response to TAA, and HLA-class II antigens are expressed on the surface of Mϕ and CD_4-T cells, generating INFγ of cytokine in activation of CTL, Mϕ and LAK.

There are many reports[26-28)] that the activation of helper T cells are an initial event of activated tumor-immunity.

In this study, it is clarified that Mϕ are activated with the help of OK-432 which is proportionate to its concentration and they exert on helper T cells as an initial event in sequence of cytocidal effects.

It is emphasized that activation of helper T cell is mandatory for stimulation of Mϕ. OK-432 plays a main role in activation of T cells and also recognition of helper T cells and CTL, for tumor-antigen is essential for step of enhanced tumorimmunity. From a result of this experimental study, it is defined that there is a close correlation between suppression of cell proliferation and HLA-class II antigen expression with help of IFNγ.

In the normal gastric mucosa, the HLA class I and II

antigens are expressed as it is consistent with the reports by Natali,[29] David[30] and Ghosh.[31] There are no good correlation among differentiation, the depth of cancer infiltration, invasive types and nodal involvement. It differs from a relationship with the prognosis in breast cancer, colon cancer and melanomas.[8, 9, 10]

On the other hand, Ferron[32] pointed out a close correlation between HLA-DR antigen expression and inflammatory cell infiltration. In this series, there is no good correlation between disease stages and HLA-DR antigen expression.

Recent studies clarified that T cells are activated with the help of MHC-antigen expression and TIL is induced with the help of HLA-DR antigen expression.[33] In fact, complicated environment exists in clinical case as the immunosuppressive state such as appearance of suppressor Mϕ. It is contemplated that HLA-DR antigen lies in the cell, not existing on the surface.[35] In such a case, it is difficult to predict activated responses of tumor-immunity. This is the reason why the degrees of TIL are not necessarily proportional to HLA-DR antigen expression.

Local OK-432 administration is of great effect in promoting HLA-class I and II antigen expressions. Even in moderately differentiated carcinomas, HLA-DR antigen was uniformly expressed.

In the case of having uniformity of HLA-DR antigen expression, TIL was increased, including Mϕ infiltration in interstitial tissues. On the basis of the result of experimental and clinical studies, it is emphasized that OK-432 plays a role in enhancement of host mediated tumor-immunity with the help of IFNγ. TIL findings well reflect activated tumor immunity.

And also it is assured that the procedures of local administration of OK-432 are of great benefit in clinical use in reflection of enhanced HLA-class I and II antigen expression.

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