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IMPAIRED TUMOR PHENOTYPES IN CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGEN-INDUCIBLE CELLS ORIGINATED FROM HUMAN LUNG ADENOCARCINOMA

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SUMMARY: The class II major histocompatibility complex (MHC) antigens play important roles in T cell activation and are thought to be involved in tumor development. Using anti-class II antibodies with interferon gamma (IFN-γ), we have here selected class II MHC antigen positive cancer cells from the human lung adenocarcinoma cell line A549, which is originally negative (>95%) for the antigens. A part of the class II antigen-positive cells presented a flat morphology, which was not observed in the parental A549 cells. Class II antigen expression in these flat cells was IFN-γ inducible; there was a correlation between the inducibility and phenotypic changes. A class II antigen-inducible flat subline restored the ability of contact inhibition and anchorage-dependent growth as well as lost tumorigenicity in athymic mice.

Class II major histocompatibility complex (MHC) antigens play important parts in the initiation of antigen-specific immune responses; they present antigenic peptides to relevant helper T (Th) cells. The Th cells recognize peptide-class II antigen complexes via their T cell receptors, and trigger various immune responses (1, 2).

Several reports have suggested that expression of class II antigens is altered in tumors, or is associated with tumor development (3, 4, 5). Transfection of class II genes to murine cancer cells results in reduced tumorigenicity (6). Inducibility of class II antigen expression by IFN-γ is shown to be related with reduction of tumorigenicity in *ras*-

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Abbreviations: MHC, major histocompatibility complex; IFN-γ, interferon-gamma; PBS, phosphate-buffered saline; FITC, fluoloscein isothiocyanate.

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transformed murine fibroblasts (7). Moreover, recent studies have revealed that genetically-engineered class II MHC antigen-positive (class II^+) tumors can stimulate protective antitumor immunity (8, 9). These observations suggest that class II antigens are useful targets for the development of antitumor immunotherapy. However, the class II^+ tumor cells in these papers have been demonstrated to be tumorigenic in athymic mice, indicating that the cells *per se* still retain their transformed phenotypes.

We show here an *in vitro* evidence implicating that class II antigen expression inducibility may be also associated with impaired tumor phenotypes of human lung adenocarcinoma A549. Our data suggest that IFN-γ inducible class II MHC antigen-positive A549 cells are able to reduce their transformed phenotypes.

MATERIALS AND METHODS

Selection of Class II MHC Antigen-Positive Human Lung Cancer Cells by Panning. The human lung adenocarcinoma cell line A549 ($1x10^5$ cells/ml) was cultured in ERDF medium (Kyokuto Seiyaku, Tokyo, Japan) supplemented with 5% fetal calf serum (FCS, Whittaker BIOPRODUCTS, Walkersville, MD, USA) and 50 IU/ml of IFN-γ (Genzyme, Cambridge, MA, USA) at 37 °C in a 95% air/5% CO_2 atmosphere for 72 hr. The cells were detached from the culture dishes using 1 mM EDTA/phosphate-buffered saline (PBS), and washed with medium for two times. Then the cells were resuspended in 50 μl of 5% FCS/ERDF medium containing 1 μg/ml of mouse anti-human MHC class II-DR antigen monoclonal antibody (PHARMINGEN, San Diego, CA, USA), and incubated on ice for 1 hr. After washing once with medium, the cells were subjected to anti-mouse IgG polyclonal antibody (Tago, Burlingame, CA, USA)-coated dishes which were prepared as described (10), and incubated at 37 °C for 2 hr. After gentle washing the dishes with medium for three times, attached cells were collected by pippeting. After two rounds of the panning procedure, the class II antigen$^+$ cells were scaled-up and sublines were cloned by standard limiting dilution.

Flow Cytometric Analysis. Cells ($1x10^6$ cells) were incubated in a staining buffer (5% FCS/PBS containing fluoloscein isochiocyanate [FITC]-conjugated mouse anti-human class II MHC-DR antigen monoclonal antibody [PHARMINGEN]) on ice for 30 min. After washing with PBS, the cells were analyzed using an Epics flow cytometer (Coulter, Hialeah, FL, USA).

Cell Growth Analysis. Cells were inoculated at a density of $1x10^5$/ml in 5% FCS/ERDF medium, and cell numbers were counted at daily intervals for two weeks. All experiments were done in duplicate.

Colony Formation Assay. The colony formation assay was performed as described (11). In brief, $1x10^3$ cells were suspended with 0.33% agarose (Sigma, St. Louis, MO, USA) dissolved in culture medium and overlayed on an agarose bed (0.5% agarose and culture medium) preliminary prepared in a 6 cm culture dish (Falcon 1007, Becton Deckinson Labware, Franklin Lakes, NJ, USA), and incubated at 37 °C in a CO_2 incubator. Colonies were scored four weeks after plating.

Tumorigenicity Assay. Four week-old nude mice (BALB/c nu/nu, Charles River Labs., Wilmington, MA, USA) were injected subcutaneously with $1x10^7$ A549 cells or

$3x10^7$ phenotypically-changed cells suspended in 300 μl of ERDF medium. Cells were scored as tumorigenic if a visible nodule (>0.5 cm of diameter) appeared at the site of injection.

RESULTS AND DISCUSSION

A549 cells are originally >95% negative for class II antigen, though only less than 5% of the cells are weakly positive. When cultured with 50 IU/ml IFN-γ, class II^+ cells has increased to 6%, indicating that A549 cells originally contain the 1% cell population whose class II antigen expression is negative but inducible by IFN-γ (data not shown). However, the rest of class II^- cells are still negative for the antigen even in the presence of IFN-γ. In order to enrich class II^+ cells from A549 cells, panning was performed in the presence of IFN-γ. After two rounds of panning procedure, recovered cells were expanded without IFN-γ. Flow cytometric analysis revealed that most of the cells were class II^+. Unexpectedly, a part of the class II^+ cells presented a flat morphology, which was not observed in original A549 cells (Fig. 1). These flat cells seemed to stop their growth at confluency, suggesting that the flat cells exhibited a contact inhibition.

To further characterize morphologically-changed cells, we next tried to clone the flat cells. Conventional limiting dilution established several flat sublines (shown in Table 1). One of the flat sublines, termed A5DC7, was used for further experiments. Flow cytometric analysis revealed that the A5DC7 cells lost the surface expression of class II antigens (Fig. 2A). As described above, A549 lung carcinoma cells originally contain a 1% fraction whose class II antigen expression is IFN-γ inducible. To examine whether the A5DC7 flat subline comes out of the 1% IFN-γ inducible population of A549 cells, cells were cultured with 50 IU/ml IFN-γ. They intensely expressed the class II MHC antigens (Fig. 2A). Other flat sublines (A5DC8, A5DM1) indicated a similar phenotype; their class II antigen expression patterns were also IFN-γ inducible (Table 1). On the other hand, constantly class II^+ sublines (A5DD4, A5DD5) neither presented a flat morphology nor contact inhibition. These results suggest that class II antigen expression *per se* is not associated with phenotypic changes. Rather, it is likely that inducibility of class II antigen expression is correlated with phenotypic changes observed in class II^+ A549 cells.

To examine whether A5DC7 cells actually cause a contact inhibition, cells were inoculated at a density of $1x10^5$ cells/ml, and cell numbers were counted at daily intervals

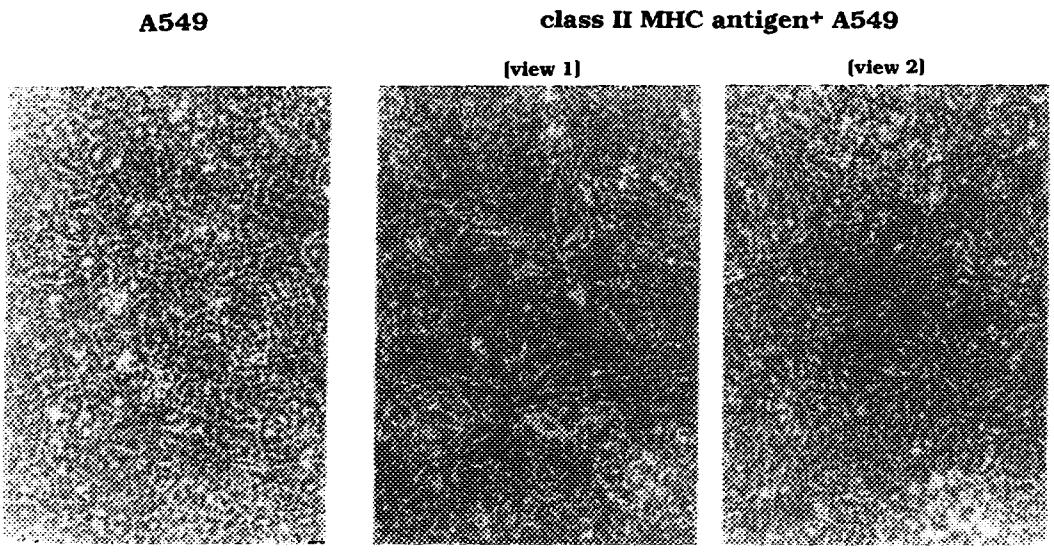

Figure 1. Morphological change and contact inhibition of a part of class II MHC antigen-positive A549 lung adenocarcinoma. Class II$^+$ cells were selected by two rounds of panning (see "MATERIALS AND METHODS") and then expanded. Magnification of each view is x 80.

for two weeks. Growth of the A5DC7 cells in a log phase is similar to that of original A549 cells; their doubling times were 24 hr. When entered a stationary phase, A5DC7 cells stopped their growth at a density of 2.7×10^6 cells/ml, while A549 cells did not (Fig. 2B). This indicates that A5DC7 cells restore the ability of contact inhibition.

In order to assess the malignancy of class II antigen-inducible A5DC7 cells, colony formation assay was performed. As shown in Table 2, A5DC7 cells completely lost the

Table 1. *Correlation between class II MHC antigen expression inducibility and phenotypic changes observed in class II$^+$ sublines originated from A549 cells*

Cell lines	Class II MHC antigen expression	Cellular phenotypes	
		Flat morphology	Contact inhibition
(original)			
A549	-	No	No
(class II$^+$ sublines)			
A5DC7	±	Yes	Yes
A5DC8	±	Yes	Yes
A5DM1	±	Yes	Yes
A5DD4	+	No	No
A5DD5	+	No	No

Class II MHC antigen expression in each cell line was analyzed by flow cytometry. +, constantly positive; -, negative; ±, negative but IFN-γ inducible. Cellular phenotypes were assessed by microscopic observations.

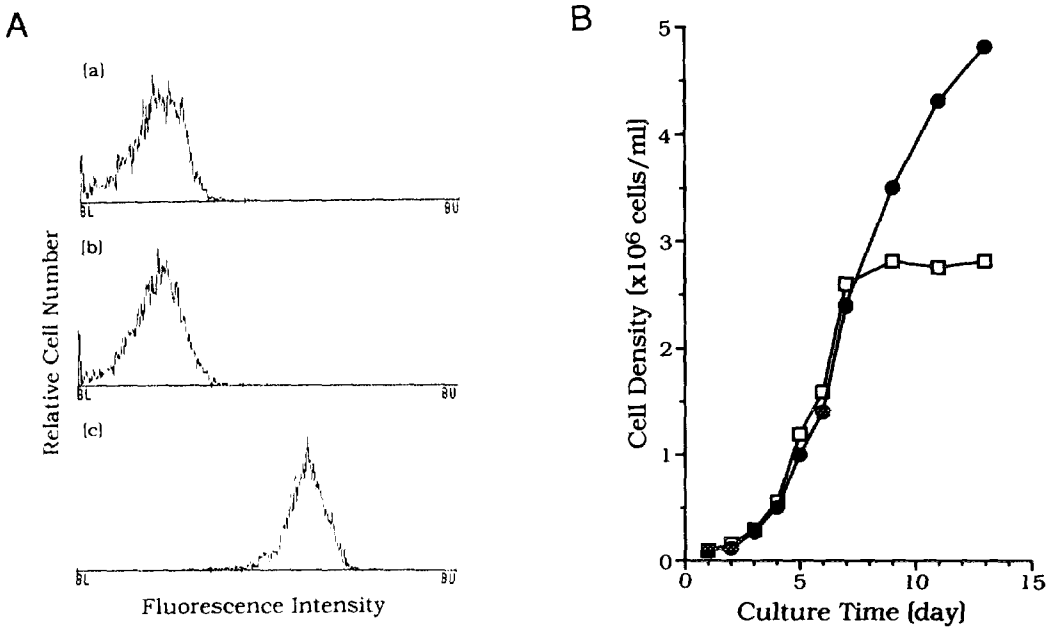

Figure 2. (A) Class II antigen expression of the morphologically changed cells is IFN-γ inducible. A subline A5DC7 cells were treated with (a) control mouse serum IgG, (b) anti-class II-DR, or (c) anti-class II-DR in the presence of 50 IU/ml IFN-γ. Then the cells were applied to flow cytometric analysis. (B) Growth curve of class II MHC antigen-inducible A5DC7 cells (open square) or parental A549 cells (closed circle). Cells were inoculated at $1x10^5$ cells/ml, and cell numbers were counted at daily intervals. Experiments were done in duplicate.

colony formation ability on agarose gels, suggesting that A5DC7 cells restore the normal anchorage-dependent growth. Microscopic analysis revealed that A5DC7 cells were in a single cell even after six weeks of inoculation (data not shown). On the other hand,

Table 2. *Growth property of parental A549 lung adenocarcinoma cells or class II MHC antigen-inducible A5DC7 cells*

Cells	Growth efficiency (%) in agarose gel[a]	Tumorigenicity in nude mice[b]	
		Tumors per injection	Latency (weeks)
A549	31, 27, 33	6/6	2 to 3
A5DC7	0, 0, 0	0/6	

[a]Colony formation assay was performed in triplicate. Growth efficiency (%) was scored as a following formula: Growth efficiency (%)=colony number/plating cell number x 100.

[b]Cells were scored as tumorigenic if a visible nodule (>0.5 cm of diameter) appeared at the site of injection. Mice that did not develop tumors were observed for 6 weeks.

parental A549 lung cancer cells formed colonies at efficiencies of 27 to 33%. To test *in vivo* tumorigenicity, $1x10^7$ of A549 cells and $3x10^7$ of A5DC7 cells were injected into athymic nude mice. A5DC7 cells were also impaired the tumorigenicity (Table 2).

Present data show a correlation between class II MHC antigen expression inducibility and loss of tumor phenotypes in A549 human lung adenocarcinoma. This also proposes a possibility that class II antigen expression inducibility might be associated with tumor suppression at least in A549 cells. A recent evidence (12) has indicated that tumor suppressor retinoblastoma protein (RB) rescues IFN-γ induction of class II antigen expression in non-inducible RB defective breast carcinoma cells, which links class II antigen expression inducibility to tumor suppression. A549 cells have been shown to contain normal RB molecule (13). Therefore, it is possible that RB or RB-activating tumor suppressive molecules, such as p53 (14), cyclin dependent kinase inhibitors (15, 16), may be up-modulated in our tumor phenotype-impaired class II MHC antigen-inducible cells.

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