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Cellular Stress- and Transformation-associated Cell Surface Antigens Expressed on Human and Rodent Tumor Cells

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Stress-induced proteins may have significant roles in anti-tumor resistance. To clarify the immunobiological roles of these proteins, we first developed monoclonal antibody (mAb) H1A that detects the HeLa cell-surface antigens whose expression was enhanced by treatment of the cells with physico-chemical stressors, such as heat, H_2O_2 and tumor necrosis factor. H1A (IgM) detects several molecules with mol. wt. 30, 43, 75, 90, 100, 120 and 150 kDa in Western blot analysis of HeLa cell lysates. Although the antigen was constitutively expressed on the HeLa cell surface, the cell-surface expression of H1A-defined antigen was rapidly enhanced (within 1 h) after heat treatment of HeLa cells. H1A antigens were also transformation-associated, since 1) the activated oncogene-transformed fibroblasts expressed the antigens, but parental nontransformed cells did not, and 2) certain human neoplastic but not normal cells strongly expressed the antigens. Furthermore, H1A mAb also partly blocked the cytotoxicity of purified protein derivatives-stimulated human T cell receptor $\gamma\delta$-type T cells towards HeLa cells. Taken together, these data indicate that H1A-defined stress-inducible proteins may play a vital role in anti-tumor resistance by cytotoxic T cells.

Key words: Tumor antigen — Cellular stress — T cell

Eukaryotic cells induce a set of proteins, so called stress proteins or hsp,[2] when they are exposed to various physical and chemical stressors, such as heat, superoxide, amino acid analogues, glucose deprivation, and certain cytokines, including TNF.[1-8] Hsp are also induced in cells infected by certain viruses.[9] Furthermore, neoplastically transformed cells frequently show enhanced expression of hsp in their cytoplasm.[9-11]

Recent findings strongly suggest that hsp may play an important role in evoking the host's T cell responses.[12, 13] It was directly demonstrated that TCR $\gamma\delta$-type T cells could respond to 65 kDa mycobacterial hsp.[14, 15] Furthermore, it appears that these particular T cell subsets interact with self hsp molecules.[14, 16-19] In fact, a recent report showed that peptide fragments of self hsp molecules could be eluted from the antigen-binding groove of HLA class I molecules.[20]

We previously demonstrated that rat 70hsp homologue could be expressed on the cell surface of an activated H-*ras* oncogene-induced transformant W31 clone, as defined by mAb #067.[21] This molecule was not expressed on the cell surface of a parental nontransformed rat fetus fibroblast WFB. This study strongly suggests that certain stress proteins are expressed on the cell surface of cells during neoplastic cell transformation. However, mAb #067 reacts species-specifically with rat cells, and it remains to be determined whether stress-induced proteins in human cells have the same profiles as seen in the rat cells.

In this paper, we developed mAb H1A, using ATP-binding proteins as the immunogen, since the majority of hsp could bind to ATP.[22, 23] This mAb detects cell-surface antigens whose expression was enhanced by various stressors, although the H1A-defined antigens did not appear to be known hsp molecules, such as 70hsp and 90-hsp. The expression of H1A-defined antigens on the cell surface seemed to be associated with cell transformation by activated oncogenes. Moreover, the data suggested that these antigens may be involved in the mechanism of PPD-stimulated TCR $\gamma\delta$-type T cell cytotoxicity towards HeLa cells.

MATERIALS AND METHODS

Animals BALB/c mice were obtained from CLEA Japan Inc., Shizuoka. In the experiment, 6- to 10-week-old male mice were used.

Cells HeLa human cervical carcinoma cell line was mainly used in the experiment. BALB3T3 cell clone #5 and its transformed clones, Bras-h and BMT-f, were obtained by transfection with activated H-*ras* and polyoma middle T DNAs, respectively, as described previously.[24]

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[2] Abbreviations used: hsp, heat-shock protein; hsc, heat-shock cognate; FACS, fluorescein-activated cell sorter; FCS, fetal calf serum; rIL-2, recombinant IL-2; mAb, monoclonal antibody; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; PPD, purified protein derivatives; TCR, T cell receptor; TNF, tumor necrosis factor.

A WKA rat fetus-derived cell, designated WFB, and its transfected clones, W14 and W31, with an activated-H-*ras* oncogene were also established as previously described.[25] The cells were cultured in Eagle's modified culture medium supplemented with 5% FCS and 292 μg/ml of L-glutamine.

Purification of ATP-binding protein from HeLa cells Approximately 5×10^8 HeLa cells at 80–90% confluency in the monolayer cell culture were incubated at 42°C for 12 h. After incubation at 37°C for 24 h, cells were lysed in HEPES buffer (20 m*M* HEPES pH 7.0, 2 m*M* Mg acetate, 25 m*M* KCl, 10 m*M* $(NH_4)_2SO_4$, 0.8 m*M* dithiothreitol, 0.2 TIU/ml Aprotinin) containing 0.5% NP-40 (Nonidet P40). Lysates were separated from the cell pellet by centrifugation at 14,000 rpm for 30 min. The lysate was passed through a 10 ml ATP-agarose column equilibrated with HEPES buffer. The column was washed with HEPES buffer with 1 *M* KCl until UV-280 had returned to the base line, and then washed once with the initial HEPES buffer. ATP-binding components were eluted wth HEPES buffer containing 1 m*M* ATP, and precipitated with 50% ammonium sulfate. The precipitates were collected by centrifugation at 15,000 rpm for 1 h, then lysed and dialyzed against HEPES buffer.

Production of mAb BALB/c mice were immunized i.p. three times with 0.5 mg of ATP-binding proteins at intervals of 14 days. Five days after the last immunization, approximately 2×10^8 immunized mouse spleen cells were fused with 4×10^7 NS-1 mouse myeloma cells by the method described previously.[25,26] The supernatants were screened for antibody activity against ATP-binding proteins by ELISA. Then the positive supernatants were screened for antibody activity against HeLa cell proteins by Western blotting and indirect immunofluorescence. After limiting dilutions, one hybridoma clone H1A with IgM isotype was selected.

Immunoblotting HeLa cells were washed three times with PBS. After resolution of 0.5% NP40 or 0.5% CHAPS (3-((3-cholamidopropyl)dimethylammonio)-1-propane sulfate) lysates of 2×10^6 HeLa cells on 8–10% SDS-PAGE gels, proteins were transferred to PVDF membranes for 2.5 h at 100 V. After saturation of non-specific binding sites with a blocking buffer (5% non-fat dry milk, 50 m*M* Tris pH 7.6), the membrane was incubated with mouse mAbs, including H1A, 7D3 (IgM) and anti-mammalian 70 kDa hsp 3a3 (IgGl) (Affinity BioReagents, Neshanic Stations, NJ), for 2 h. Then the membrane was washed 3 times in PBS containing 0.1% Tween 20, and incubated with peroxidase-labeled goat anti-mouse IgG/IgM antibody for 1 h. After vigorous washing, the blots were detected by using ECL detection reagents (RPN2105, Amersham). We usually exposed the films to these reagents for 10 to 60 before development.

Stress treatment of cells Cells at approximately 80% confluency in the monolayer cell culture were incubated for 30 min at 42°C, for 30 min with 20 μM H_2O_2, and for 10 h with 5 U/ml of recombinant human TNF (2×10^7 U of specific activity/mg; Genzyme, Boston, MA) in culture medium at 37°C. Cells were rinsed twice with the medium and cultured for various periods at 37°C. These treatments did not affect the cell viability. Cells were then analyzed for antigen expression in a FACS analyzer.

FACS analysis of cells and immunohistochemical study of tissues stained with mAb The cells were washed once with ice-cold PBS containing 0.05% NaN_3, and approximately 2×10^6 cells in 0.5 ml of PBS/NaN_3 were incubated with a saturating amount of mAb for 40 min at 4°C. Cells were washed with PBS/NaN_3 and incubated with FITC-conjugated goat anti-mouse IgG/IgM for 40 min at 4°C. Cells were washed and fixed, and samples were run on the FACS analyzer. Immunohistochemical analysis was also performed on frozen preparations of neoplastic and nonneoplastic regions of certain tissues. The procedure was described elsewhere.[25]

Cytotoxicity of TCR $\gamma\delta$-type enriched T cells against HeLa In order to assess whether H1A-defined antigens could participate in the cytotoxicity of TCR $\gamma\delta$-type T cells towards tumor cells, PPD-reactive TCR $\gamma\delta$-type T cells were enriched by depleting CD4 (+) and CD8 (+) T cells, as previously described.[27] Briefly, PBL were cultured for 7 days in the presence of 10 μg/ml of PPD (Japan BCG Inc., Tokyo). Dead cells were removed by Ficoll-Hypaque density centrifugation, and live cells were further cultured for 3 to 5 days in the presence of 10 μg/ml of PPD and 350 U/ml of rIL-2 (Shionogi Pharmaceutical Co., Tokyo). The cells were reacted with anti-CD4 and anti-CD8 mAbs for 30 min at 4°C, washed with PBS, and reacted with iron beads-conjugated goat anti-mouse IgG (Advanced Magnetics Inc., Cambridge, Mass.). CD4 (+) and CD8 (+) T cells were then removed by a Biomag separator (Advanced Magnetics Inc.). The T cells enriched in CD4 (−) and CD8 (−) cells were washed with PBS twice, and used in the cytotoxicity assays. Prior to the cytotoxicity assays, the purity of TCR $\gamma\delta$-type T cells was assessed by using mAb TCR δ-1 (T Cell Sciences, Cambridge, Mass.). The cytotoxicity of these T cells against H1A antigen-positive and negative target cells, such as HeLa and K562, respectively, was evaluated by using ^{51}Cr at 50 and 100 E/T ratio, as described elsewhere.[26] In the case of HeLa cells, heat-treated and non-treated cells were employed. In the blocking study, the effect of mAb H1A on the cytotoxicity towards HeLa cells was assessed. HeLa cells were treated with saturating amounts of H1A or a control 7D3 mAb at 4°C for 60 min. The cells were washed twice with PBS, and used in the cytotoxicity assays.

RESULTS

H1A could detect 30, 43, 75, 90, 100, 120 and 150 kDa molecules in the immunoblotting experiment Seven hybridomas were picked up after screening by ELISA and Western blotting. Only one clone, designated as H1A, could produce an antibody targeting cell surface molecules. In the immunoblotting experiments shown in Fig. 1a, mAb H1A could detect seven molecules, 30, 43, 75, 90, 100, 120 and 150 kDa in HeLa cell lysate. Furthermore, the immunoblotting data show the same reactivity pattern of mAb H1A in NP40 and CHAPS (a milder detergent) lysates. While it is perfectly possible that a family of related proteins is being recognized, it is also possible that the minimal use of proteolytic enzyme inhibitors in preparing the lysates might have allowed degradation to occur. To rule out this latter possibility, the same lysate was assessed to determine whether 70hsp was degraded by using anti-70hsp mAb 3a3. As shown in Fig. 1b, 3a3 detects only the 70hsp molecule, suggesting that the antigens recognized by H1A are not proteolytic artefacts but a family of related proteins. There was also no difference as to the mobility of proteins between non-reduced and reduced forms (data not shown), indicating that no intermolecular disulfide bonds are present in H1A-defined antigens. Furthermore, H1A mAb did not react with purified bovine 72hsp/73hsc (StressGen, Victoria, Canada) or human 90hsp (StressGen) in an immunoblotting experiment (data not shown). These data suggest that H1A could not detect classical 70hsp and 90hsp.

Stress treatment increased expression of H1A-defined antigens on the cell surface The change of cell surface expression was examined by FACS analysis. As shown in Fig. 2, the espression of H1A-antigens was increased after various kinds of stress treatments, such as 42°C heat shock (Fig. 2A), hydrogen peroxide (Fig. 2B) and TNF (Fig. 2C). As a stress-non-inducible antigen, we used MHC class I molecule, as detected by HH-1 mAb, which reacts with a nonpolymorphic determinant of MHC class I molecule. The expression of this antigen was not influenced by heat treatment (Fig. 2D), indicating that the enhancement of the cell-surface expression of H1A-antigen was not an artefact. The expression of H1A-antigens on the cell surface was slightly increased at 1 h after the heat shock, and reached the maximum level at 48 h after the heat shock (Fig. 3). It then decreased gradually and returned to the pre-shock level at 96 h after the heat shock (data not shown).

H1A-antigens are transformation-associated molecules expressed on the cell surface Cell surface expression of

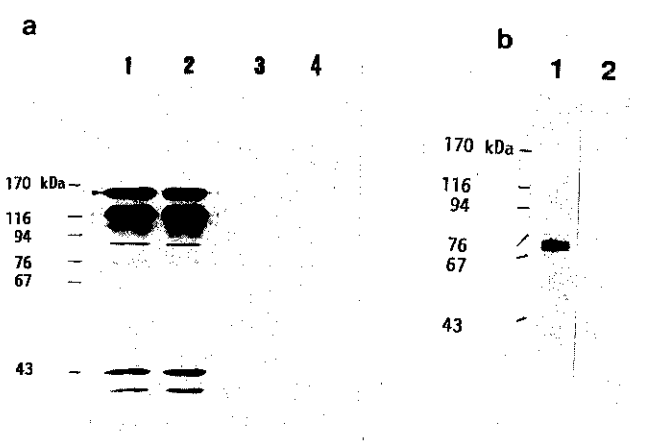

Fig. 1. a) Western blot analysis of H1A-defined antigens in HeLa cells. Cell lysates with NP40 (lanes 1 and 3) and CHAPS (lanes 2 and 4) were subjected to 8% SDS-PAGE. The antigens were transferred to the PVDF membrane, and the reactivity of H1A mAb (lanes 1 and 2) and a control 7D3 mAb (lanes 3 and 4) was assessed. The antigens were detected by using ECL detection reagents as described in "Materials and Methods." b) Western blot analysis of the reactivity of anti-70hsp mAb 3a3 to the same HeLa cell lysate as used in a). Lanes 1 and 2 were reacted with mAbs 3a3 and 7D3, respectively.

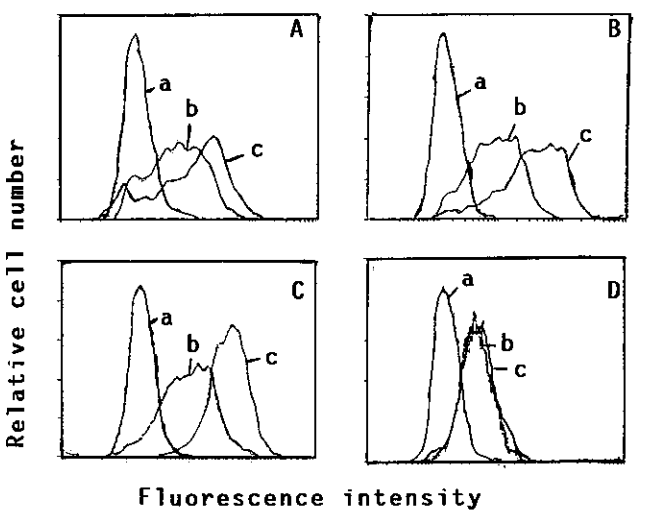

Fig. 2. Stress inducibility of H1A-defined antigens evaluated by FACS. HeLa cells were treated with heat (A), hydrogen peroxide (B) and TNF (C) as described in "Materials and Methods." Then, the cells were cultured for another 24 h, and the cell surface expression of H1A-defined antigens was studied. (D) shows the reactivity of heat-treated HeLa cells with HH-1 mAb, which detects a nonpolymorphic determinant of MHC class I molecule. a, second (goat anti-mouse Ig conjugated with FITC) antibody alone; b, constitutive (non-stress) expression; c, stress-induced expression.

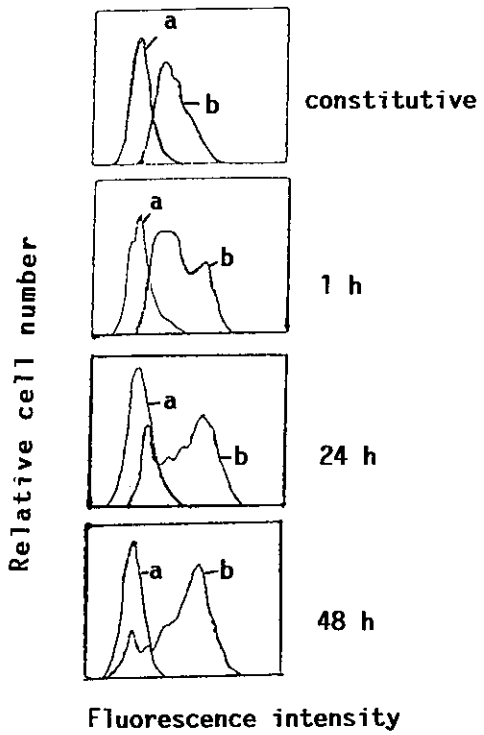

Fig. 3. FACS analysis of the expression kinetics of H1A-defined antigens. HeLa cells were treated for 30 min at 42°C, and were cultured for 1, 24 and 48 h at 37°C. Then, H1A mAb reactivity to these cells was assessed by FACS. a, second antibody alone; b, stress-induced expression.

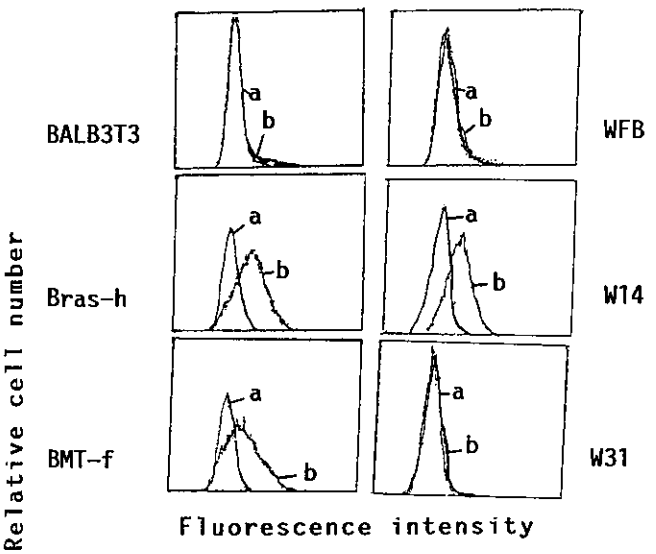

Fig. 4. FACS analysis of the constitutive expression of H1A-defined antigens on oncogene-induced transformants of mouse BALB3T3 and rat WFB fetal fibroblasts. a, second antibody alone; b, H1A reactivity to the cells.

H1A-antigens was analyzed in various kinds of cell lines. MAb H1A could detect the antigens of murine cells as well as human cells. As shown in Fig. 4, murine fibroblast cell lines, BALB3T3 and WFB, which have characteristics similar to those of non-transformed cells, do not express H1A-antigens on the cell surface. The cytoplasmic expression of H1A antigens in BALB3T3 and WFB was also undetectable, as assessed by an indirect immunofluorescence technique (data not shown). In contrast, Bras-h and BMT-f, which are EJ-ras- and polyoma middle T oncogene-transformed clones, respectively, clearly expressed H1A-antigens on their cell surface. W14, an EJ-ras-transformed WFB clone, also expresses the antigens. However, another WFB transformant, W31, could not express the H1A-antigens, although this clone showed a strong expression of antigens in the cytoplasm (data not shown).

Cytoplasmic expression of H1A antigens is enhanced in neoplastic tissues We assessed the immunohistochemical reactivity of frozen sections of normal human adult tissues, including lung, heart, liver, spleen, pancreas, kidney, esophagus, stomach, intestines, lymph nodes and PBL. H1A did not react with any of these tissues, except monocytic lineage cells present in the spleen, lymph nodes and PBL. We also investigated immunohistochemically some human cancer tissues. As shown in Fig. 5a, H1A strongly reacts with the cell cytoplasm of cer-

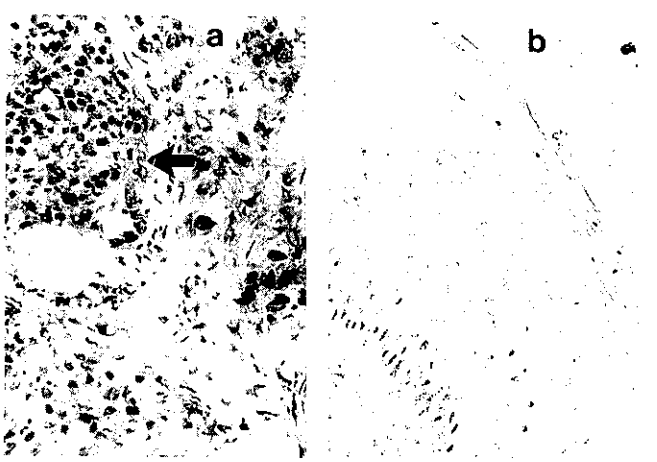

Fig. 5. Immunohistochemical analysis of H1A-defined antigens in the human cervical carcinomas. a, neoplastic tissue (arrow indicates positive findings); b, nonneoplastic tissue. ×200.

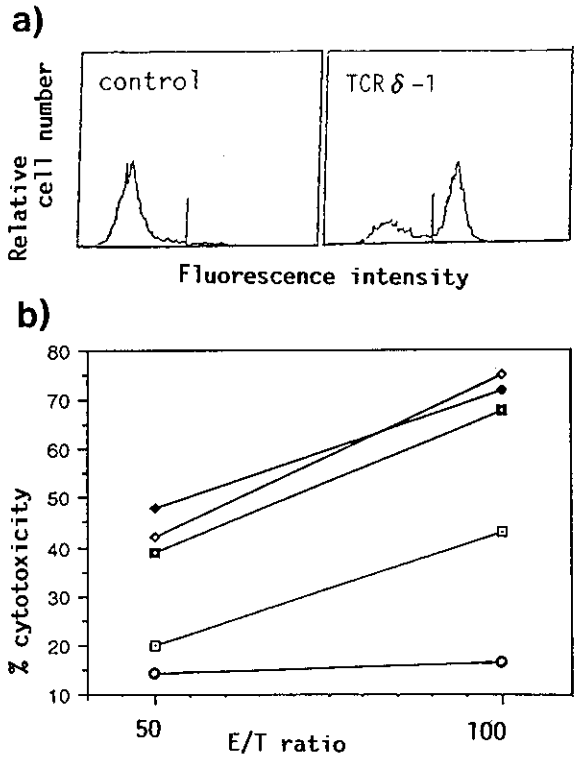

Fig. 6. FACS pattern and cytotoxicity of TCR $\gamma\delta$-type-enriched T cells against HeLa cell targets. a) FACS pattern of the reactivity of TCR δ-1 mAb with effector T cells. PBL were stimulated by PPD, and proliferated T cells were treated with a saturating amount of anti-CD4 and anti-CD8 mAbs. CD4 (−) and CD8 (−) T cells were enriched by depleting CD4 (+) and CD8 (+) T cells with magnetic beads-conjugated anti-mouse Ig. Then, the reactivity of TCR δ-1 mAb to CD4 (−) and CD8 (−) T cells was studied by FACS. b) Cytotoxicity of TCR $\gamma\delta$-type-enriched T cells towards ^{51}Cr-labeled HeLa cell targets with (◆) or without heat treatment (■), and K562 target without heat treatment (○). Inhibition of the T cell cytotoxicity by H1A (□) and 7D3 (◇) mAbs against ^{51}Cr-labeled HeLa cell targets without heat treatment at 50 and 100 E/T ratio.

tain squamous carcinoma cells of the uterine cervix. The reactivity with noncancerous squamous cells was weaker than that with neoplastic cells (Fig. 5b), although it appeared that nuclei are stained with almost the same intensity. Three out of five cases examined in this study showed almost the same reactivity pattern. This was true for the esophageal squamous cell carcinomas. H1A also reacted with some of the renal cell carcinomas and breast carcinomas (data not shown). The cell-surface expression of H1A antigens in these neoplastic tissues was not determined, since it is difficult to isolate fresh tumor cells free from the tissues.

H1A-antigens may participate in the cytotoxicity of TCR $\gamma\delta$-type enriched T cells towards HeLa cells Because the above data suggest that H1A-antigens are tumor-associated antigens, we examined whether H1A-antigens may be involved in the mechanism of T cell cytotoxicity towards H1A antigen-positive HeLa cells. Recent reports have indicated that some hsp may interact directly with TCR $\gamma\delta$-type T cells. Hence, we enriched CD4 (−) and CD8 (−) T cells from PPD-stimulated allogeneic PBL by depleting CD4 (+) and CD8 (+) T cells with magnetic beads-conjugated anti-mouse antibodies. As shown in Fig. 6a, we could enrich TCR $\gamma\delta$-type T cells to approximately 80% purity as assessed by TCR δ-1 mAb. This partially purified T cell population was still responsive to PPD, since the cells required the presence of PPD to proliferate *in vitro* (data not shown). Fig. 6b shows that these T cells very efficiently lyzed H1A antigen-positive HeLa cell targets, whereas they were not cytotoxic to H1A antigen-negative K562 tergets. Heat treatment of HeLa cells did not affect the susceptibility of HeLa cells to these T cells. However, H1A but not a control 7D3 mAb could in part inhibit the cytotoxicity of these T cells towards non-stressed HeLa cells, suggesting that H1A-antigens may participate in the cytotoxic action of TCR $\gamma\delta$-type T cells.

DISCUSSION

Previously, we found that 70 kDa stress-inducible proteins could be expressed on the cell surface of a rat fetal fibroblast WFB which was transformed by the activated H-*ras* oncogene.[21, 28] This indicates that certain transformation-associated antigens belong to the hsp family. Our preliminary data also suggested that these protein-positive transformants generate strong protective immunity in syngeneic animal hosts. Therefore, hsp could play an important role in antitumor immunity. In other words, the host immune system may recognize these proteins as strong immunogenic targets not only in states of infection and autoimmunity,[12, 13, 17, 18] but also in antitumor protective immunity.

In order to clarify this interesting point, we attempted to develop mAbs which react with stress-inducible antigens on the cell surface of HeLa cells, by using ATP-agarose-separated hsp-rich proteins as an immunogen to mice for hybridoma production. Our data indicated that mAb H1A-defined antigen expression was highly enhanced by physico-chemical stressors. By western blot analysis using HeLa cells, H1A was shown to interact with a family of related molecules of 30, 43, 75, 90, 100, 120 and 150 kDa. These molecules are not proteolytic artefacts, since anti-70hsp mAb 3a3 showed only one band which corresponds to 70hsp. There was also no obvious change in the intensity of protein blots between

heat-treated and non-treated HeLa cell NP40 lysates (data not shown). Since H1A detects 75, 90 and 100 kDa molecules, we examined whether H1A could react with classical 70hsp and 90hsp, by using purified bovine 72-hsp/73hsc and human 90hsp. The data indicated that H1A did not react with these proteins (data not shown). On the other hand, it is known that isoforms of 70hsp and 90hsp proteins, such as 78Bip and 94grp, are induced by Ca^{2+} ionophore and glucose starvation.[29-31] Hence the possibility that mAb H1A detects these proteins remains to be examined.

So far, immunoprecipitation experiments using H1A have not been successful. Therefore, we do not know which molecules among the H1A-defined antigens detected in the western blot are important for the cell-surface expression. However, our preliminary data suggested that 90, 100 and 120 kDa molecules might be expressed on the cell surface, since cells such as BALBMC, which expresses H1A antigens on the cell surface, showed these three molecules in western blot analysis (data not shown).

To our knowledge, no report demonstrating that hsp are definitely expressed on the cell surface has been published. However, several studies including our previous report have strongly suggested that hsp may be expressed on the cell surface.[3, 21, 32, 33] It is well known that hsp play important roles as molecular chaperones.[34] A recent report also indicated that the hydrophobicities of MHC class I and 73hsc are very similar,[35] although there is only 24% homology in primary amino acid sequence between these two proteins. Perhaps heat shock, as well as other treatment which induce classical hsp expression, also increases surface expression of H1A-defined antigens.

Our data indicated that the cytoplasmic and cell surface expression of H1A antigens is closely associated with cell transformation. In rat WFB and mouse BALB-3T3 cells, H1A antigens were expressed in the cell cytoplasm and on the surface of only oncogene-induced transformed cells, but not of parental WFB and BALB-3T3 cells. Even in W31 cells, the cytoplasmic constitutive expression of H1A antigens was clearly demonstrated, although cell surface expression could not be detected. Furthermore, W31 cells did not express H1A antigens on the cell surface even after stress treatments such as heat, hydrogen peroxide and TNF (data not shown). These findings suggest that a regulatory mechanism for the cell-surface expression of H1A-defined antigens exists in the transformed cells.

In the immunohistochemical analyses of human tissues, although the cell-surface expression was not determined, the cytoplasmic expression of H1A antigens appeared to be associated with the neoplastic conversion of squamous cell epithelium. In fact, H1A antigens were detected in many squamous cell carcinomas, but only weakly in noncancerous squamous cells of the same patients.

Recently, an increasing number of reports has shown that T cells interact with hsp.[14, 15, 36, 37] We also found that certain squamous cell carcinomas are frequently accompanied with infiltrating TCR $\gamma\delta$-T cells. Accordingly, we investigated whether H1A antigens may participate in anti-tumor responses by the hosts, particularly in the cytotoxicity of TCR $\gamma\delta$-T cells towards tumor cells expressing H1A antigens. Our data indicated that H1A antigens are most likely involved in the cytotoxicity of $\gamma\delta$-T cells towards HeLa cells, although it was not certain that H1A antigens could participate as target antigens for $\gamma\delta$-type TCR. The blocking capability of H1A mAb was not strong. The blocking effect of anti-CD3 mAb on the cytotoxicity of $\gamma\delta$-T cells was also not strong (data not shown). However, these results seemed to be due to the polyclonality of effector T cells, and perhaps due to the LAK-like activity that may be induced by the relatively high concentration of IL-2 used in proliferation of these T cells. Furthermore, there was no obvious difference in susceptibility to the cytotoxicity between HeLa cells with or without heat treatment. This fact may also be attributed to the possibility that H1A antigens in HeLa cells are expressed constitutively. The constitutive level of H1A antigen expression may be enough for efficient interaction with TCR $\gamma\delta$-T cells. Moreover, H1A mAb did not react to PPD in Western blot analysis (data not shown). Although H1A mAb detects only natural form of PPD epitope rather than denatured epitope in Western blot analysis, the H1A epitope may not be present on PPD molecules. Therefore, one can consider that some epitope(s) in H1A antigen other than H1A epitope may be important in $\gamma\delta$-T cells-H1A antigen interaction.

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