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Production and Characterization of a Monoclonal Antibody against Surface Glycoprotein, gp61, on K562 Erythroleukemia Cells

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A multipotential hematopoietic cell line, K562 cell, was differentiated into megakaryocyte by a chemical inducer, PMA, with an enhanced expression of gpIIIa accompaning with a distinct morphological change. On the other hand, K562 cells were differentiated into erythrocytes by other chemical inducers, DMSO or butyrate, with a concomitant increase in hemoglobin accumulation. An antigen of apparent molecular weight of 61 kDa was identified on the surface of K562 cells by using monoclonal antibody raised against K562 cells. The antigen was considered to be a glycoprotein molecule rich in sialic acids and the epitope of antigen was sensitive to neuraminidase digestion or peroxidase oxidation, but resistant to heat treatment. The 61 kDa surface antigen was increased or decreased in its expression along differentiation of K562 cells into megakaryocytes or erythrocytes, respectively.

KEY WORDS: Monoclonal Antibody, K562 Erythroleukemia Cells, Surface Antigen, Cell Differentiation

New insights into the regulation of hematopoiesis have been obtained by the study of established culture of erythroleukemia cells which can be induced to differentiate *in vitro*. Depending on the agents employed, the erythroleukemia cell line, K562 cell, can be induced to differentiate along a variety of pathways including erythroid, megakaryocytic, monocytic, and lymphoid lineages (Anderson *et al.*, 1979; Honma *et al.*, 1989; Tetteroo *et al.*, 1984). Differentiation toward a given lineage can be monitored by a reduction of growth potential, an altered expression of genes such as *c-myc*, *c-sis* and *c-fos*, translocation of protein kinase C from the cytoplasm to the membrane and an induction of surface markers specific to the lineages (Colamonici *et al.*, 1986; Alitalo *et al.*, 1990; Hocevar *et al.*, 1992).

A lot of cell surface molecules are thought to play a major role in complex biological events such as differentiation, development and adhesion and invasion of tumor cells. Most of the effort to date has been devoted to defining and characterizing the cell surface molecules which are involved in these cellular events. A number of surface antigens including integrins and their ligands were characterized to participate in the cellular regulation (Hynes, 1987). Although the expression of the surface molecules is highly regulated in response to growth factors and

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cytokines (Heino *et al.*, 1989; Dedhar, 1989; Defilippi *et al.*, 1991), viral infection and chemical treatments (Plantefaber and Hynes, 1989; Dedhar and Saulnier, 1990) and during hematopoietic differentiation (Hickstein *et al.*, 1988; Hickstein *et al.*, 1989; Tetteroo *et al.*, 1984), little is known regarding the molecular mechanisms by which the expression of the molecules is regulated.

We have now employed the K562 cells as a model system to dissect the molecular mechanisms controlling the hematopoiesis and malignancy. An exposure of K562 cells to phorbol 12-myristate 13-acetate (PMA) causes megakaryocytic differentiation (Tetteroo *et al.*, 1984; Bütler *et al.*, 1990), whereas an exposure to hemin, sodium butyrate, or dimethyl sulfoxide (DMSO) enhances hemoglobin synthesis and induces differentiation along an erythroid lineage (Anderson *et al.*, 1979). In the present study, cell surface-marker molecule of K562 cells was identified and characterized by the monoclonal antibody technique and its role was investigated in the artificial differentiation of K562 cells.

Materials and Methods

Cell culture

K562 erythroleukemia cells and NS1 myeloma cells were obtained from the Aichi Cancer Center Research Institute, Nagoya, Japan. NS1 myeloma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), 10% fetal calf serum (FCS) and K562 cells were cultured in Rosewell Park Memorial Institute medium 1640, plus penicillin, streptomycin and 10% FCS at 37° C in an incubator flushed continuously with 5% CO_2.

Production of monoclonal hybridoma cells

Monoclonal antibodies (MAbs) against K562 cells were produced by the hybridoma technique as described by Kennett (1988). Six week-old female Balb/c mice were immunized by intraperitoneal injection of intact K562 cells repeatedly in an interval of 1 week. On the 3rd day after the final injection, the spleen was removed and many holes were poked and spleen cells were prepared from the organ by perfusing Hank's balanced salt solution with a syringe. The spleen cells were suspended in 5 ml of ice-cold 0.17 M NH_4Cl solution and put aside for 10 min and then centrifuged to obtain a pellet of cells. The spleen cells and NS1 cells suspended in 5 ml DMEM separately were mixed in a round bottomed centrifuge tube and centrifuged. The cell pellet was resuspended in 0.2 ml of 40% polyethylene glycol (PEG) 1000 in DMEM and followed by centrifugation 6 min at 1000 rpm. To the pellet, 5 ml DMEM without serum was added so as to disperse the pellet and DMEM with 20% FCS was then added to the cell suspension. The fused cells were cultured and hybridoma clones began to appear on the 6th day after fusion.

Hybridoma cells were screened for the production of antibodies against K562 cells by enzyme-linked immunosorbent assay (ELISA) and subcloned by limiting dilution and successively in semi-solid agarose as described by Kennett (1988).

Enzyme-linked immunosorbent assay

K562 cells (10^5 cells per well) were attached by 0.01% poly L-lysine onto 96-well flat-bottom polystyrene microtiter plates and fixed by 0.25% glutaraldehyde. The bottom of each well was coated with 0.1% BSA in PBS and washed three times with PBS/0.05% Tween 20, and 50 μl of hybridoma culture media was added and the plates were incubated for 2 h at 37°C. After washing, 100 μ of 1/500 diluted goat anti-mouse IgG-peroxidase conjugate was added to each well and the plates were then incubated for 2 h. After washing five times, 200 μl of substrate solution (10 mg O-phenylenediamine, 4 μl of 30% H_2O_2 solution in 10 ml of 0.1 M citrate buffer, pH 4.5) was added to each well. 175 μl of reaction mixture from each of wells was transferred to a clean plate and the absorbance was measured at 450 nm with a multiscan photometer (Titertek Multiskan, Flow Laboratories).

SDS-polyacrylamide gel electrophoresis and Western blot analysis

Cultured cells were washed with PBS and then solubilized in sodium dodesyl sulfate (SDS)-

Laemmli sample buffer. The proteins were analyzed by one dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described in Laemmli (1970). Western blotting was carried out basically as described by Towbin *et al.* (1979). The proteins separated on SDS-PAGE gels were transferred to nitrocellulose paper. After incubation in 3% BSA in PBS, the paper was treated with the primary antibody and then alkaline phosphatase-conjugated anti-mouse IgG antibody. After extensive washing, color development of the blots was performed.

Chemical and heat treatments

For peroxidate oxidation, K562 cells were incubated with 10 mM metaperiodate for 1 h at room temperature. Neuraminidase digestion of the cells was conducted by using 0.1 unit/ml of the enzyme solution at 17°C for 2 h. For the test of heat stability of the surface antigens, cells were incubated at 100°C for 15 min and the binding of antibody to both pretreated and post-treated cells was analyzed by ELISA.

Radiolabeling and immunoprecipitation

Exponentially growing K562 cells were incubated with [^{35}S]-methionine (10 μCi/ml) in methionine-free medium overnight. The labeled cells were washed with PBS three times, solubilized in a RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.5) containing 0.1% NaN_3, and used for immunoprecipitation. Labeled cell lysates were centrifuged at 3,000 rpm in a microcentrifuge, and supernatant was incubated with monoclonal antibody-bound protein A Sepharose for 1 h. The immunoprecipitate was dissolved in SDS sample buffer under reducing conditions and separated electrophoretically using 10% SDS-PAGE. After SDS-PAGE, the gel was stained, subjected to autoradiography and exposed at -70°C to Kodak RP X-Omat film.

Hemoglobin assay

After K562 cells were treated with differentiation-inducing agents for 4 days, the amount of hemoglobin in the cells was measured by the photometric method using benzidine. 5 to 10 μl of cell extract was mixed with 0.2 ml of 1% benzidine in 90% acetic acid, and then 0.2 ml of 1% H_2O_2 freshly prepared in water from a 30% stock solution was added. After 20 min, the assay mixtures were diluted with 2 ml of 10% acetic acid, and the optical density was measured at 515 nm within 30 min. Human hemoglobin (Sigma Chemical Co.) was used as a standard.

Immunofluorescence microscopy

To determine the expression of gpIIIa, K562 cells were washed with phosphate-buffered saline, attached on cover slips with poly-L-lysine (Sigma Chemical Co.) and fixed in 3.7% formaldehyde for 30 min. Staining was performed using fluorescein isothiocyanate-conjugated gpIIIa MAb (DAKO Corp.) diluted 1:200 for 1 h in the dark. After staining, specimens were analyzed with a confocal imaging system using a 400 × objective lens with 3 zoom (Carl-Zeiss LSM410 Invert).

Results

Morphological change of differentiation inducer-treated K562 cells

Various differentiation-inducing agents were treated separately to K562 cells at density of 5 × 10^5 cells/ml to activate the cellular differentiation. The K562 cell morphology was changed by the addition of 100 nM PMA, 60 μM hemin or 1 mM sodium butyrate. According to a microscopic examination, the cells became slightly larger and more adhesive to the plastic bottom of the culture flask. An immediate cell growth arrest was also happened after exposure to each of chemical (data not shown). However, in the case of sodium butyrate, the cell growth resumed gradually on 2nd day of butyrate treatment. Thus, K562 cell differentiation was accompanied by an arrest of growth, a change in morphology, and an increased adherence of the cells.

Changes of gpIIb/IIIa and hemoglobin synthesis during K562 cell differentiation

Erythroid differentiation of K562 cells can be monitered by the accumulation of hemoglobin together with the expression of erythrocyte

specific surface antigens such as glycophorins, whereas megakaryocytic differentiation can be demonstrated by the increased expression of megakaryocyte-specific antigen, gpIIb/IIIa and the concomitant disapperance of hemoglobin. To analyze hemoglobin accumulation in the course of cell differentiation, the cells were cultured in the medium containing 10-100 nM PMA, 1.8% DMSO, 1 mM sodium butyrate, or 60 μM hemin. On the 3rd and 6th day of PMA addition, no significant change in hemoglobin content was detected as verified by benzidine peroxide assay. In contrast to PMA treatment, the medium containing 1.8% DMSO, or 1mM sodium butyrate promoted significantly hemoglobin accumulation in the cells. Most notable hemoglobin accumulation was observed in the cells cultured in the medium containing 60 μM hemin for 6 days (Fig. 1).

In order to determine whether PMA treatment induces the megkaryocytic differentiation, the expression of gpIIIa, a megakaryocyte-specific integrin, was examined by direct immunofluorescent staining using anti-gpIIIa MAb. As shown in Fig. 2, gpIIIa was extensively expressed on the surface of PMA-treated K562 cells.

These results clearly indicate that the exposure of K562 cells to PMA induces megakaryocytic differentiation, wherease the exposure to hemin, sodium butyrate, or DMSO causes differentiation along an erythroid lineage.

Changes in total proteins during K562 cell differentiation

As K562 cells were differentiated to megakaryocyte or erythrocyte by various inducers, changes in total cellular protein patterns were examined by SDS-PAGE. Even though the overall protein pattern on the gel was similar in the course of cell differentiation towards two differnt lineages, the expression of 200 KDa protein was slightly enhanced in PMA-treated cells, whereas the protein level was markedly decreased following erythroid differentiation by DMSO or sodium butyrate (Fig. 3).

Production of monoclonal antibody against K562 cell surface proteins

In order to identify the K562 cell surface-specific antigens, monoclonal antibodies against K562 cells were produced by the hybridoma technique as described in "Materals and Methods". The rate of successful fusion seemed to be affected by composition and pH of PEG solution, and temperature, time of PEG treatment (data not shown). The hybridomas were maintained in HAT medium for 2 weeks after the fusion (Fig. 4). The culture media were screened for specific antibody

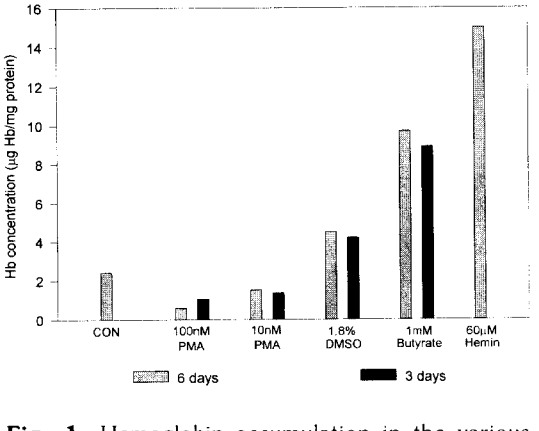

Fig. 1. Hemoglobin accumulation in the various differentiation inducer-treated K562 cells. Hemoglobin contents were determined by the benzidine-peroxide assay on 3rd and 6th day after the inducer addition described in "Materials and Methods".

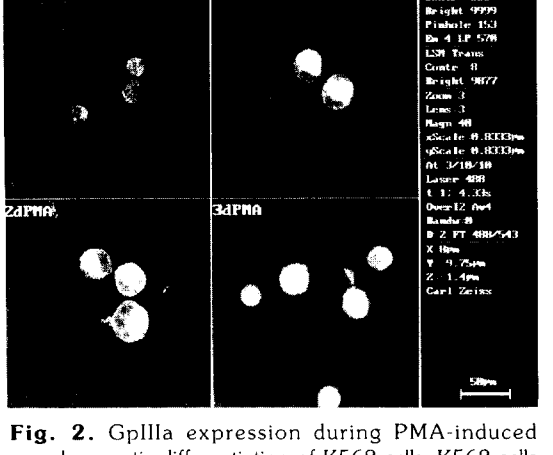

Fig. 2. GpIIIa expression during PMA-induced megakaryocytic differentiation of K562 cells. K562 cells were treated with 100 nM PMA and gpIIIa expression was then examined by immunofluorescence microscopy using anti-gpIIIa antibody. × 400.

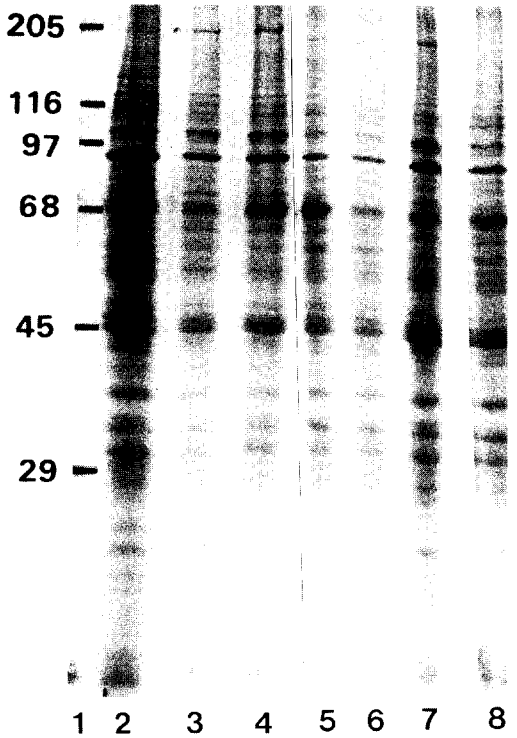

Fig. 3. Changes in total proteins during K562 cell differentiation. K562 cells treated with 10-100 nM PMA, 1.8% DMSO or 1 mM sodium butyrate for either 3 days (lanes 7-8) or 6 days (lanes 2-6) and the cellular proteins (50g/lane) were analyzed by SDS-PAGE. Lane 1, molecular weight markers (myosin (205 KDa), β-galactosidase (116 KDa), phosphorylase b (97 KDa), bovine serum albumin (68 KDa), and ovalbumin (45 KDa)); lane 2, control K562 cells; lane 3, 100 nM PMA; lane 4, 10 nM PMA; lane 5, 1.8% DMSO, lane 6, 1 mM sodium butyrate; lane 7, 100 nM PMA; lane 8, 1 mM sodium butyrate-treated K562 cells.

production by direct solid-phase ELISA on fixed K562 cells and negative control cell lines. Clones that reacted with K562 cells were further screened for antibody specificity and subcloned by limiting dilution periodically. Thirteen positive clones were obtained and among 13 clones, the culture media of EK-2 clone showed strong and specific reactivity against K562 cells on the ELISA test (Table 1).

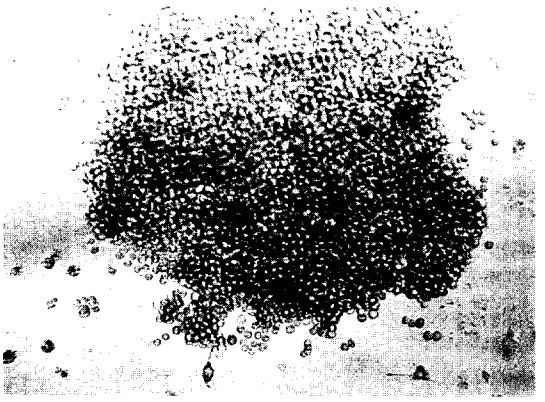

Fig. 4. Microscopic photograph of EK-2 nonoclonal hybridoma cells. The clone was one of hybridomas formed by the fusion of spleen cells and NS-1 cells on two weeks after the fusion. × 100.

Table 1. Specificity of EK-2 antibody. Reactivities were measured against various hematopoietic cell lines by ELISA with undiluted or diluted supernatant obtained from EK-2 monoclone culture.

Cell line	Dilution		
	1	10	100
NS-1 (negative control)	–	–	–
K562 (chronic myeloid leukemia)	+++	+	–
HL-60 (acute promyelocytic leukemia)	+	–	–
SK-LY-16 (human Burkitt's lymphoma)	–	–	–

Identification and biochemcial characterization of the antigen recognized by EK-2 antibody

For the identification of antigen recognized by EK-2 antibody, K562 cells were metabolically labeled with [^{35}S]-methionine and the labeled cell lysates were immunoprecipitated by using EK-2 antibody and the immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography. As shown in Fig. 5, EK-2 antibody immunoprecipitated a single polypeptide with an apparent molecular weight of 61 KDa when the protein was running on the SDS-PAGE under

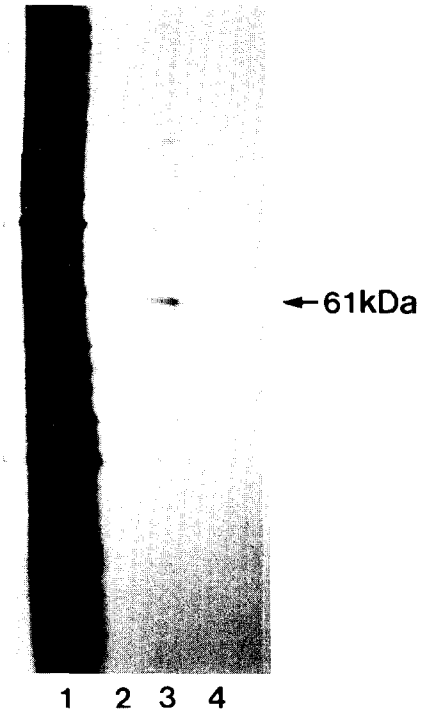

Fig. 5. Immunoprecipitation of [^{35}S]-methionine labeled K562 cellullar proteins by EK-2 antibody. [^{35}S]-methionine labeled K562 cells were dissolved in RIPA buffer and centrifuged and the resulting supernatant was immunoprecipitated with EK-2 antibody as described in "Materials and Methods". The precipitated proteins were analyzed by SDS-PAGE and autoradiography. Lane 1, [^{35}S]-methionine labeled K562 cellular proteins; lane 2, no antibody; lane 3, EK-2 antibody; lane 4, preimmune serum.

reducing condition.

In order to examine whether the EK-2 antigen is a glycoprotein, K562 cells were pretreated with periodate or neuramidase and the binding capacity of EK-2 antibody on the target antigen was then analyzed. As shown in Table 2, the antigen-binding capacity of EK-2 antibody was significantly diminished in the periodate- or neuraminidase-treated cells. Neuramidase or periodate treatment data suggests that the carbohydrate moiety may constitute an important part of the epitope for EK-2 antibody.

Heat stability of the antigen was also tested, and it was found that the binding properties between EK-2 antibody and antigenic determinants on the target cells were rather resistant to heat treatment

Table 2. Sensitivities of EK-2 antigens to heat, peroxidate oxidation or neuraminidase degradation. K562 cells were treated with heat or chemicals and then attached to the bottom of 96 well assay plate and EK-2 antibody binding capacities to the cells were measured by ELISA.

	*Absorbance obtained with treatment
PBS (Control)	0.935
Heat	0.729
Periodate Oxidation	0.360
Neuramidase	0.134

* Mean of duplicated ELISA tests

(Table 2). Inferring from its heat stability and sensitivity to periodate oxidation or neuraminidase degradation, the target determinant recognized by EK-2 seems to be carbohydrate moiety, especially rich in sialic acid.

Expression of EK-2 antigen during K562 cell differentiation

To examine whether the expression of EK-2 antigen is regulated during K562 cell differentiation, PMA, DMSO or sodium butyrate was treated to K562 cells to induce the megakaryocytic or erythroid cell differentiation and then the cellular proteins were analyzed by Western blotting using EK-2 antibody. As shown in Fig. 6, immunoblotting experiments indicated that the antigen expression was enhanced as K562 cells differentiated into megakaryocytic lineage, whereas its expression disappeared during the differentiation into erythrocytes. Therefore, we tentatively conclude that the epitope of EK-2 antibody would be one of the specific antigenic determinants present on the K562 cell itself and be highly expressed as the cells differentiate along the megakaryocytic lineage.

Discussion

K562 cells can be regarded as pluripotent hematopoietic progenitor cells expressing markers for erythroid, granulocytic, monocytic, or megakaryocytic lineages as defined by surface-

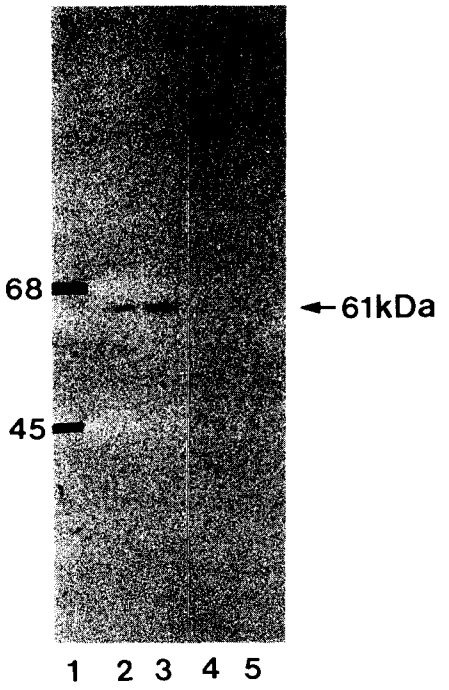

Fig. 6. Quantitative changes of the EK-2 antigen during K562 cell differentiation. The K562 cells were treated with each of various inducer for 6 days and the cell lysates were analyzed by SDS-PAGE and immunoblotting using EK-2 hybridoma supernatant. Lane 1, molecular weight marker (bovine serum albumin (68 KDa) and ovalbumin (45 KDa)); lane 2, control K562 cells; lane 3, 100 nM PMA; lane 4, 1.8% DMSO; lane 5, 1 mM sodium butyrate-treated K562 cells.

antigen expressions. In the present study, it was confirmed that treatment of the cells with PMA could induce differentiation along a megakaryocytic lineage, whereas treatment of hemin, sodium butyrate or DMSO induce the erythroid differentiation. PMA treatment of K562 cells led to an almost immediate growth arrest accompanied by a change in cell morphology: the PMA-treated cells became slightly larger and flatter than untreated cells and became adherent to the plastic of the culture flasks; untreated K562 cells could be resuspended by shaking the culture flask whereas the PMA-treated cells had to be scraped off using an utensil for the preparation of suspension.

PMA-treated cells enhanced the expression of 200 KDa protein, whereas DMSO- or sodium butyrate-treated cells reduced its expression. The 200 KDa protein might be a marker for differentiation along the megakaryocytic or erythroid lineages. Giltay *et al.* (1988) reported that differentiation of K562 cells along the megakaryocytic pathway is associated with markedly enhanced cell surface expression of the gpIIb/IIIa integrin (Fig. 2) which is unique to platelets and megakaryocytes and which serves as a receptor for several ligands including fibrinogen, fibronectin, von Willebrand factor, and vitronectin. However Zutter *et al.* (1992) have also shown that megakaryocytic differentiation of K562 cells is accompanied by expression of the $\alpha_2\beta_1$ integrin, a cell surface collagen receptor, and acquisition of the ability to adhere to collagen substrates. 200 KDa protein or 61 KDa EK-2 antigen whose synthesis is enhanced by PMA treatment seems to be distinct from the two integrins mentioned above judging from high discrepancy in the molecular weights.

Monoclonal antibodies are the most highly selective yet versatile of biochemical isolation tools. Their usefulness in identification and isolation of a particular molecule contained in an extremely complex mixture is unsurpassed. Therefore, in this study mouse monoclonal antibody was also produced against K562 cell line in order to detect antigens associated with differentiation or malignancy of K562 cells.

One hybridoma clone, producing IgG antibody and reactive predominantly with K562 cell line, was selected, established and designated as EK-2. In order to begin biochemical characterization of the antigen, immunoprecipitation and treatment of cells with reagents capable of destroying specific classes of determinants were performed. The results of these studies indicate that the MAb EK-2 binds to an antigen with an apparent molecular weight of 61 KDa. The antigen activity was resistant to heat treatment, but periodate or neuraminidase treatment partially destroyed the antigenic activity. The fact that the epitope is sensitive to a neuraminidase digestion or a perodiodate oxidation but unaffected by heat treatment suggests that the epitope for EK-2 is saccharides in nature. Due to the possibility that this antigen is a glycoprotein, and the anomalous electrophoretic behavior of glycoproteins during

SDS-PAGE, this molecular weight value (61 KDa) can only be used for comparison with other antigens analyzed by this procedure.

The expression of EK-2 antigen highly increased as the cells differentiated along megakaryocytes, whereas the antigen had a tendency of depletion as the cells differentiated along erythrocytes. In view of the high frequency of EK-2 antigen expression on K562 cells themselves or K562-derived megakaryocytes, and its non-expression on K562 derived erythrocytes, the EK-2 MAb may serve as a specific immunological probe to study the molecular mechanism of differentiation and malignancy of hematopoietic cells.

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K562 적혈구암 세포주의 표면 당단백질에 대한 단클론항체의 생성 및 특성

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K562 적혈구암 세포는 phorbol 12-myristate 13-acetate(PMA)에 의해서 대핵세포로 분화되고 gpIIIa의 증가, megakaryocyte와 유사한 형태학적 변화로 특징지워진다. 또한 K562 세포는 dimethyl sulfoxide(DMSO)나 butyrate와 같은 화학적 유도원에 의해 적혈구로 분화가 유도되고 동시에 헤모글로빈이 축적된다. 본 연구에서는 K562 세포에 대한 단클론항체를 생성하고 이를 이용하여 61 KDa의 표면항원을 동정하였다. 단클론항체 EK-2에 의해 인지되는 61 KDa의 표면항원은 sialic acid가 풍부한 당단백질로 사료되고, 그 epitope는 neuraminidase 절단과 peroxidase oxidation에 민감하며, 열처리에는 안정하다. K562 세포의 대핵세포로 분화시에는 61 KDa 표면항원의 발현은 증가하며, 적혈구로 분화시에는 그 발현이 감소한다. EK-2 단클론항체는 조혈세포의 분화 및 암화과정의 분자적 수준을 연구하기 위한 면역학적 probe로 이용 가능할 것으로 기대되어진다.